



LUND UNIVERSITY

Real-time PCR for diagnosis of botulism and quantification of neurotoxin gene expression in *Clostridium botulinum*

Artin, Ingrid

2008

[Link to publication](#)

Citation for published version (APA):

Artin, I. (2008). *Real-time PCR for diagnosis of botulism and quantification of neurotoxin gene expression in Clostridium botulinum*. [Doctoral Thesis (compilation), Division of Medical Microbiology]. Division of Medical Microbiology.

Total number of authors:

1

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Real-time PCR for diagnosis of botulism and quantification of neurotoxin gene expression in *Clostridium botulinum*

Ingrid Artin

Department of Laboratory Medicine, Lund
Division of Medical Microbiology
Faculty of Medicine
Lund University
Sweden, 2008

Akademisk avhandling

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorexamen i medicinsk vetenskap kommer att offentligen försvaras i Patologens föreläsningssal, Sölvegatan 25, Lund, fredagen den 29 februari 2008, kl 13.15 Fakultetsopponent, Docent Miia Lindström, Institutionen för livsmedels- och miljöhygien, Veterinärmedicinska fakulteten, Helsingfors Universitet, Finland.

Academic thesis

Which by due permission of the Faculty of Medicine at Lund University will be publicly defended for the degree of Doctor of Philosophy on Friday the 29th of February 2008, at 13.15, at Sölvegatan 25, Lund.

The Faculty opponent is Miia Lindström, Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Finland.

Abstract

The objectives of the work presented in this thesis have been to develop molecular methods for (i) detection of *Clostridium botulinum* in food and clinical samples, and (ii) monitoring the expression of the neurotoxin gene, *cnt*, during different growth phases and under different growth conditions.

For the diagnosis of botulism, a real-time PCR-based method including an internal amplification control for *C. botulinum* types A, B and E was developed. The method was used to diagnose the first case of wound botulism in an injecting drug user in Sweden, which was also the first case of wound botulism reported to be caused by *C. botulinum* type E. It was previously assumed that only proteolytic *C. botulinum* could cause infectious botulism. However, this case revealed that wound botulism can be caused by nonproteolytic *C. botulinum*.

When developing new food products it is important to consider not only the occurrence and quantity of pathogens in the food chain, but how the processing and subsequent storage and handling will affect them. Especially for food designed to have a long shelf-life, understanding the influence of environmental factors, food preservatives, and type of packaging on microbial growth and virulence expression is very important.

Quantitative reverse transcription PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to monitor the neurotoxin expression in *C. botulinum* types A, B, and E. The relative *cnt* expression varied with growth phase in all three types, reaching a maximum as the late exponential phase was changing into stationary phase. However, for the proteolytic type A, a second increase could be seen in late stationary phase in contrast to type E. The same behaviour has earlier been observed for proteolytic *C. botulinum* type B with a second increase in *cntB* mRNA when the bacteria reached the death phase. For the nonproteolytic *C. botulinum* type E the *cntE* expression declined rapidly after the peak in the transition between exponential and stationary phase. The *cntE* mRNA half-life was calculated to be approximately nine minutes.

When investigating the effect of carbon dioxide on growth and neurotoxin expression, we found that while proteolytic type A was not affected in either regard, the nonproteolytic types B and E were. Their growth was slowed, but the relative *cnt* expression was increased with an elevated CO₂ concentration, with fivefold higher levels of *cntB* mRNA and type B toxin being detected at 70% CO₂ than at 10%. For type E the increase in expression at 70% CO₂ was two-fold greater, when compared with 10% CO₂. For the proteolytic strain Hall A microarrays

were also used to follow the genome-wide expression profiles. Comparison between the results from the qRT-PCR and the microarrays shows that both methods seem valid, as the results are similar. For example, the expression of the positive regulator gene, *cntR*, followed the same pattern as *cntA*, but at a much lower level.

In conclusion, our findings, confirmed with both qRT-PCR and ELISA, shed a new cautionary light on the potential risks of botulism associated with nonproteolytic *C. botulinum* and the use of modified atmosphere packaging. In addition, our PCR method was successfully used on clinical samples and proved to be a valuable complement to standard methods.

Table of contents

| | |
|---|----|
| Abstract | |
| List of papers | |
| Abbreviations | |
| <i>Clostridium botulinum</i> | 1 |
| The organism..... | 1 |
| The toxin | 6 |
| The disease | 9 |
| Genetics..... | 12 |
| Diagnostics and detection methods | 14 |
| Diagnostic PCR..... | 16 |
| Real-time PCR..... | 17 |
| Internal amplification control..... | 20 |
| Detection limits | 22 |
| Selectivity..... | 22 |
| Types of samples..... | 23 |
| Neurotoxin expression during growth..... | 24 |
| Reverse transcription PCR (RT-PCR)..... | 24 |
| Real-time PCR quantification | 25 |
| Microarrays | 27 |
| Neurotoxin gene expression profiles..... | 29 |
| Effect of CO ₂ | 33 |
| Modified atmosphere packaging | 33 |
| Effects of carbon dioxide on growth, toxin gene expression, and production of neurotoxin | 35 |
| Concluding remarks | 39 |
| Acknowledgements | 41 |
| References | 43 |

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.

Paper I

Artin, I., P. Björkman, J. Cronqvist, P. Rådström and E. Holst (2007).

First Case of Type E Wound Botulism Diagnosed Using Real-time PCR.

Journal of Clinical Microbiology **45**(11): 3589-94.

Paper II

Lövenklev, M., I. Artin, O. Hagberg, E. Borch, E. Holst and P. Rådström (2004).

Quantitative Interaction Effects of Carbon Dioxide, Sodium Chloride, and Sodium Nitrite on Neurotoxin Gene Expression in Nonproteolytic *Clostridium botulinum* Type B.

Applied and Environmental Microbiology **70**(5): 2928-34.

Paper III

Artin, I., A. T. Carter, E. Holst, M. Lövenklev, D. R. Mason, M. W. Peck, and P. Rådström.

Effects of Carbon Dioxide on Neurotoxin Gene Expression in Non-Proteolytic *Clostridium botulinum* Type E.

Applied and Environmental Microbiology. Submitted for publication.

Paper IV

Artin, I., D.R. Mason, J. Schelin, M.W. Peck, E. Holst, P. Rådström, and A.T. Carter.

Effect of Carbon Dioxide on Growth and the Transcriptome of Proteolytic *Clostridium botulinum* Type A.

Manuscript.

Abbreviations

| | |
|-----------------|---|
| ANTP | associated non-toxic proteins |
| ASM | American Society for Microbiology |
| BoNT | botulinum neurotoxin |
| cDNA | complementary DNA |
| CFS | cerebrospinal fluid |
| <i>cnt</i> | botulinum neurotoxin gene |
| <i>cntR</i> | regulatory protein gene |
| CO ₂ | carbon dioxide |
| Ct | threshold cycle |
| ECL | electrochemiluminescence |
| ELCA | enzyme-linked coagulation assay |
| ELISA | enzyme-linked immunosorbent assay |
| EMG | electromyography |
| FDA | US Food and Drug Administration |
| FRET | fluorescence resonance energy transfer |
| GMO | genetically modified organisms |
| HA | hemagglutinating proteins |
| <i>ha</i> | hemagglutinating protein gene |
| HC | heavy chain |
| IAC | internal amplification control |
| KH | Henry's law's constant |
| LC | light chain |
| MAP | modified atmosphere packaging |
| mRNA | messenger ribonucleic acid |
| MS | mass spectrometry |
| N ₂ | nitrogen |
| NSF | N-ethylmaleimide-sensitive fusion protein |
| NTNH | non-toxic non-hemagglutinating proteins |
| <i>ntnh</i> | non-toxic non-hemagglutinating protein gene |
| O ₂ | oxygen |
| OD | optical density |
| ORF | open reading frame |

| | |
|------------------|---|
| p47 | 47 kDa protein in neurotoxin cluster |
| pCO ₂ | partial pressure of CO ₂ |
| PCR | polymerase chain reaction |
| qRT-PCR | quantitative RT-PCR |
| RT-PCR | reverse transcription PCR |
| SNAP-25 | synaptosomal-associated protein of 25 kDa |
| SNARE | soluble NSF-attachment protein receptor |
| VAMP | vesicle-associated membrane protein |

Clostridium botulinum

The organism

In 1820 Justinius Kerner described an outbreak where 76 people suffered from descending flaccid paralysis after eating smoked sausage. He called the disease “botulism” after the Latin word for sausage, *botulus*. Two years later he published a monograph on the “sausage poison” which contained a clinical evaluation and summary of 155 cases, including post-mortems, as well as a description of his experiments in purifying the toxin and administering it to animals. He deduced that the toxin worked by interrupting signal transmission in the muscles but not the sensory signal transmission, comparing it to rust in an electrical conductor. He even went so far as to test the toxin on himself (Erbguth and Naumann, 1999).

In 1895 Emile van Ermengem isolated a bacteria from salted ham that had caused an epidemic, with the symptoms described by Kerner, in Ellezelles (van Ermengem, 1979). What he found was gram-positive anaerobic spore-forming slightly curved rods with oval subterminal spores that he named *Bacillus botulinus*. The organism was later renamed *Clostridium botulinum*. Furthermore, he found that the disease was not caused directly by the bacteria themselves, but by a heat-labile toxin.

Later studies have shown that *C. botulinum* are motile bacteria with peritrichous flagellae and that their size varies in the range $0.3\text{-}0.7 \times 3.4\text{-}7.5 \mu\text{m}$. *C. botulinum* exhibits great cultural, physiological, and genetic diversity. Today these bacteria are known to produce seven types of botulinum neurotoxins (BoNTs), based on the serological specificity of their toxins, designated A-G. Four phenotypic groups (I-IV) can be found that differ so much that they could be considered different species (Suen et al., 1988b). Group I strains producing BoNT types A, B, and F are proteolytic and mesophilic with an optimal growth temperature of 35-40°C and a minimum growth temperature of 10°C. The spores are highly heat-resistant (Hatheway, 1993). Group II strains producing toxin types B, E, and F are nonproteolytic psychrotrophs with an optimal growth temperature of 30°C or below and a minimum growth temperature of 3°C (Graham et al., 1997). The spores have low heat resistance and, since the cells lack endogenous proteolytic enzymes for toxin activation, the toxicity can be increased by trypsinisation (Hatheway, 1993).

For both group I and II the toxin gene is generally considered to be chromosomal, though recently two strains have been found where the toxin genes were located in a plasmid (Marshall et al., 2007). Group III consists of BoNT types C and D, and they are generally

nonproteolytic. They have an optimal growth temperature of 40°C and a minimum growth temperature of 15°C. The spores have an intermediate heat resistance and the toxicity gene is phage-mediated (Eklund and Poysky, 1974). Group IV consists of type G strains and are proteolytic, asaccharolytic, and lipase-negative, have an optimal growth temperature of 37°C and spores with an intermediate heat resistance. The toxicity is enhanced by trypsinisation (Hatheway, 1993). The BoNT gene is located on a plasmid (Eklund et al., 1988; Zhou et al., 1995).

Group I and II are known to cause botulism in humans, usually from BoNT types A, B, and E, while group III (types C and D) are considered animal pathogens. Group IV (type G) has not been associated with disease, but has been found in necropsy of humans who died suddenly of an unidentified cause (Schiavo et al., 1994) and in cases reported as sudden infant death (Sonnabend et al., 1985).

The standard definition of *C. botulinum* includes all organisms known to produce BoNTs and thereby able to cause botulism in humans or animals (Prévot, 1953). However, strains have been found that are phenotypically and genetically *C. butyricum* and *C. baratii* that produce type E and F toxin respectively (Suen et al., 1988b), and *C. botulinum* type G is sometimes considered to be a different species, *C. argentinense* (Suen et al., 1988a).

C. botulinum is considered to be a ubiquitous soil bacterium, but the type of spores present varies between locations and soil type. In the western United States, especially west of the Rocky Mountains, mainly type A spores are found, generally in neutral to alkaline soil with a low organic content. In the eastern United States type B spores predominate, mainly in slightly acidic soil with a higher organic content. Type E is mainly found in connection to water, e.g. in the Great Lakes area, in shore soil, and sediment. In inland soil in Europe type B spores dominate, except in Sweden, where mainly type E is found (Dodds, 1993). The variations between different locations and types of food can be seen in Table 1, where some recent cases of foodborne outbreaks of botulism are summarised.

Table 1. Recent cases of foodborne outbreaks of botulism

| Year | Country | Food implicated | Cases | Toxin type* | Reference |
|-------------|----------------|--|--------------|-------------------------|--------------------------|
| 1989 | UK | Hazelnut purée added to yoghurt | 27 | B | (Brett, 1999) |
| 1989 | USA | Garlic-in-oil | 3 | A | (Morse et al., 1990) |
| 1991 | Sweden | Vacuum-packed hot-smoked rainbow trout | 2 | E | (Anonymous, 1991) |
| 1992 | USA | Uneviscerated salt-cured fish product (moloha) | 4 | E | (CDC, 1992) |
| 1993 | Italy | Roasted eggplant in oil | 7 | B | (CDC, 1995b) |
| 1993 | USA | Commercial canned cheese sauce | 8 | A | (Townes et al., 1996) |
| 1994 | USA | Baked potato | 30 | A | (Angulo et al., 1998) |
| 1994 | USA | Beef stew | 1 | A | (CDC, 1995a) |
| 1994 | Sweden | Hot-smoked salmon | 3 | nd | (Öberg, 1994) |
| 1994 | Sweden | Home-preserved fish | 1 | nd | (Öberg, 1994) |
| 1994 | China | Salted, fermented paste of soybeans and wax gourds | 6 | <i>C. butyricum</i> / E | (Meng et al., 1997) |
| 1995 | Italy | Canned macrobiotic food | 1 | Ab | (Franciosa et al., 1997) |
| 1995 | Canada | Seal oil | 1 | E | (Proulx et al., 1997) |
| 1995 | Canada | Fermented seal meat | 3 | E | (Proulx et al., 1997) |
| 1995 | Canada | Fermented walrus meat | 5 | E | (Proulx et al., 1997) |
| 1995 | Canada | Fermented seal meat | 1 | E | (Proulx et al., 1997) |
| 1996 | Italy | Mascarpone cream cheese | 8 | A | (Aureli et al., 2000) |
| 1996 | India | Sevu | 34 | <i>C. butyricum</i> / E | (Chaudhry et al., 1998) |
| 1996 | USA | Home-preserved tomato sauce with meat | 1 | B | (Shapiro et al., 1997) |

Table 1. Continued

| Year | Country | Food implicated | Cases | Toxin type | Reference |
|-------------|----------------|--|--------------|-----------------------|---------------------------|
| 1997 | USA | Home-pickled eggs | 1 | B | (CDC, 2000) |
| 1997 | Finland | Vacuum-packed hot-smoked whitefish | 2 | E | (Korkeala et al., 1998) |
| 1997 | Norway | Rakfisk | 4 | E | (Kuusi et al., 1999) |
| 1997 | Argentina | Home-cured ham | 6 | E | (Rosetti et al., 1999) |
| 1997 | Iran | Locally made cheese | 27 | A | (Pourshafie et al., 1998) |
| 1998 | UK | Bottled mushrooms | 2 | B | (Brett, 1999) |
| 1998 | Thailand | Home-canned bamboo shoots | 13 | A | (CDC, 1999) |
| 1998 | Denmark | Commercially produced vegetable pie | 1 | B | (Poulstrup et al., 1999) |
| 1999 | Finland | Whitefish eggs | 1 | E | (Lindström et al., 2004) |
| 2000 | France | Home-canned asparagus | 9 | B | (Abgueuen et al., 2003) |
| 2001 | USA | Fermented beaver | 7 | E | (CDC, 2001) |
| 2001 | Canada | Fermented salmon roe | 2 | E | (Dawar et al., 2002) |
| 2001 | Canada | Fermented salmon roe | 2 | E | (Dawar et al., 2002) |
| 2001 | USA | Canned tuna / Spagetti noodles and meat sauce ^a | 1 | <i>C. baratii</i> / F | (Harvey et al., 2002) |
| 2001 | USA | Frozen chili | 16 | A | (Kalluri et al., 2003) |
| 2002 | USA | Muktuk from beached whale | 8 | E | (CDC, 2003) |
| 2002 | South Africa | Tinned fish in tomato sauce | 2 | A | (Frean et al., 2004) |
| 2003 | Denmark | Garlic in chili oil | 1 | B | (Lohse et al., 2003) |
| 2003 | UK / Poland | Sausage | 2 | B | (McLauchlin et al., 2006) |

Table 1. Continued

| Year | Country | Food implicated | Cases | Toxin type | Reference |
|-------------|----------------|---------------------------------|--------------|-------------------|---------------------------|
| 2004 | UK | Hummus | 1 | nd | (McLauchlin et al., 2006) |
| 2005 | Turkey | Roasted canned mushrooms | 5 | nd | (Cengiz et al., 2006) |
| 2005 | UK | Home-preserved pork | 1 | B | (McLauchlin et al., 2006) |
| 2005 | USA | Home-salted fish | 5 | E | (Sobel et al., 2007) |
| 2006 | Thailand | Home-canned bamboo shoots | 163 | A | (CDC, 2006b) |
| 2006 | USA | Commercial carrot juice | 4 | A | (CDC, 2006a) |
| 2006 | USA | Home-prepared fermented tofu | 2 | A | (CDC, 2007b) |
| 2006 | Finland | Vacuum-packed smoked whitefish | 1 | E | (Lindström et al., 2006b) |
| 2006 | Austria | Barbecued pork ^b | 5 | nd | (Meusburger et al., 2006) |
| 2007 | USA | Commercially canned chili sauce | 5 | A | (CDC, 2007a) |

* *C. butyricum* / E – Type E toxin produced by *C. butyricum*.

C. baratii / F – Type F toxin produced by *C. baratii*.

nd – Toxin type not determined.

^a Toxin found in both, not certain if this was true at time of consumption.

^b No *C. botulinum* or toxin could be isolated

The toxin

Botulinum toxins are produced in complexes with associated non-toxic proteins (ANTP). These can include non-toxic non-hemagglutinating proteins (NTNH) and hemagglutinating components (HA). The size of the complexes varies between 300 and 900 kDa, depending on both intrinsic and extrinsic factors (Johnson and Bradshaw, 2001). Their function is not clear, but probably relate to protecting the toxin and helping it stay active after ingestion. The BoNTs are zinc endopeptidases (Schiavo et al., 1992), which share the amino acid sequence with the consensus sequence common to other Zn^{2+} -dependent metallopeptidases (Ahnert-Hilger and Bigalke, 1995). All the BoNTs bind one zinc atom per molecule, except BoNT/C, which binds two (Humeau et al., 2000). They are synthesised as single-chain 150 kDa polypeptides, and then cleaved by proteolytic nicking within a surface-exposed loop at the LC-H_N junction by bacterial or tissue proteases to give the active toxin.

The di-chain molecule consists of a light chain (LC) pharmacodynamic part of 50 kDa and a heavy chain (HC) pharmacokinetic part of 100 kDa that are linked by a disulfide bond (Ahnert-Hilger et al., 1995). The heavy chain consists of two 50 kDa domains. The C-terminal domain of the HC (H_C) is the ganglioside-binding domain. It binds to target cell membrane and internalises the toxin molecules. The binding is highly selective, binding only to gangliosides with more than one neuraminic acid residue, which is only found in high concentrations in nerve cell membranes. The toxin is internalised by the nerve cell while forming a ternary complex with the ganglioside and the receptor protein. Internalisation takes place by receptor-mediated endocytosis involving clathrin-coated vesicles (Ahnert-Hilger et al., 1995). H_N is the translocation domain, which allows the LC to escape from the endosomal compartment into the cytosol (Bade et al., 2004). It forms ion channels in lipid bilayers and is most active at endosomal pH.

The H_N domain has a pair of long (105 Å) amphipathic helices, resembling coiled coils of viral proteins, and a “translocation belt”, a long loop wrapped around the LC (Turton et al., 2002). In this way the H_N masks the catalytic cleft of the LC when the disulfide bridge is intact (Humeau et al., 2000), possibly to protect it from auto-cleavage (Ahmed et al., 2001). A comparison between the loop of BoNT/A and SNAP-25 shows both spatial and sequence homology, suggesting a mimicking behaviour between the loop and the initial recognition of SNAP-25 (Chen et al., 2007). The light chain is the zinc-dependent endopeptidase and contains the catalytic zinc atom and a conserved HExxH motif. The catalytic site, with negative surface charge, is located in a deep cleft (20 Å), which is accessible through a

channel. In order for the LC to pass through the channels formed by the HC there needs to be an acid pH in the *cis* side, like in the endosome, and a reducing environment in the *trans* side, like in the cytosol (Koriazova and Montal, 2003). It is thought that the LC partially unfolds forming a molten globule structure (Bade et al., 2004) at endosomal pH in order to pass through the HC channel, which has a diameter (~15 Å) smaller than the folded LC (55 Å × 55 Å × 62 Å) (Lacy et al., 1998). When it has passed through to the cytosol, it refolds at the interface and dissociates from the HC (Koriazova et al., 2003). This reduction of the disulfide bond is necessary for intracellular action during intoxication (Humeau et al., 2000).

Once inside the cytosol the protease can start cleaving one of the pre-synaptic proteins. These are SNAP-25 (synaptosomal-associated protein of 25 kDa), synaptobrevin (or VAMP – vesicle-associated membrane protein), and syntaxin. These are collectively referred to as SNAREs and form a synaptic fusion complex, which is a heterotrimeric, parallel four-helix bundle, that mediates the fusion of a vesicle to its target membrane (Sutton et al., 1998). Only uncomplexed or partially assembled SNARE proteins can be cleaved by the neurotoxin.

SNAP-25 is specifically cleaved by BoNT/A and BoNT/E at two distinct carboxyl terminal sites. BoNT/A cleaves Gln¹⁹⁷-Arg¹⁹⁸ and BoNT/E cleaves Arg¹⁸⁰-Ile¹⁸¹ (Schiavo et al., 1993a). Synaptobrevin is cleaved by BoNT/B at Gln⁷⁶-Phe⁷⁷ (Schiavo et al., 1992), BoNT/D at Lys⁵⁹-Leu⁶⁰ (Yamasaki et al., 1994), BoNT/F at Gln⁵⁸-Lys⁵⁹ (Schiavo et al., 1993b), and by BoNT/G at Ala⁸¹-Ala⁸² (Schiavo et al., 1994). BoNT/C cleaves syntaxin at Lys²⁵³-Ala²⁵⁴ (Schiavo et al., 1995) and can also cleave SNAP-25 (Humeau et al., 2000). The mechanism of action can be seen in Figure 1.

For a human weighing 70 kg the lethal dose of crystalline type A toxin has been estimated to be 0.09-0.15 µg intravenously or intramuscularly, 0.7-0.9 µg inhalationally and 70 µg orally (Arnon et al., 2001).

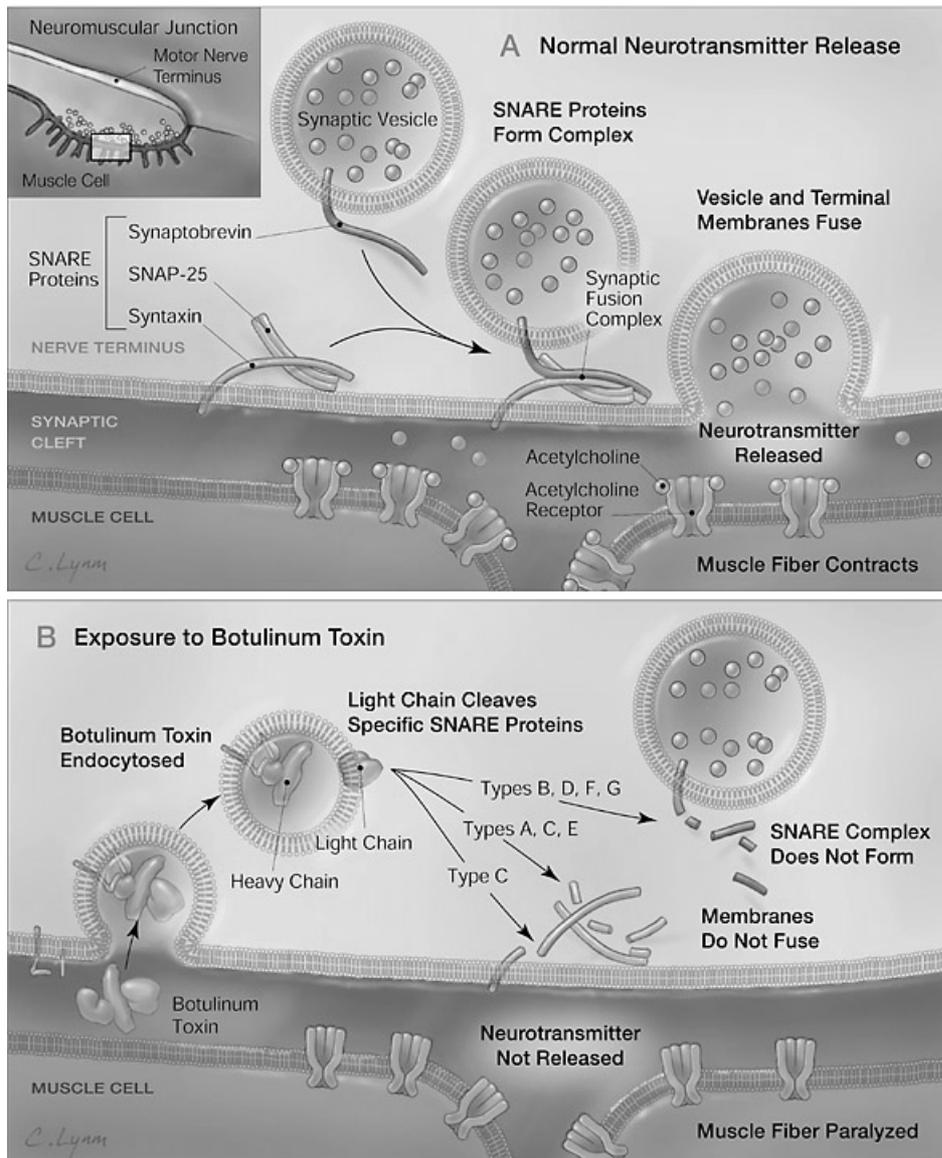


Figure 1 Mechanism of action of botulinum toxin (Arnon et al., 2001). Copyright JAMA.

A. Release of acetylcholine at the neuromuscular junction is mediated by the assembly of a synaptic fusion complex that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal cell membrane. The synaptic fusion complex is a set of SNARE proteins, which include synaptobrevin, SNAP-25, and syntaxin. After membrane fusion, acetylcholine is released into the synaptic cleft and then bound by receptors on the muscle cell.

B. Botulinum toxin binds to the neuronal cell membrane at the nerve terminus and enters the neuron by endocytosis. The light chain of botulinum toxin cleaves specific sites on the SNARE proteins, preventing complete assembly of the synaptic fusion complex and thereby blocking acetylcholine release. Botulinum toxins types B, D, F, and G cleave synaptobrevin; types A, C, and E cleave SNAP-25; and type C cleaves syntaxin. Without acetylcholine release, the muscle is unable to contract. SNARE indicates soluble NSF-attachment protein receptor; NSF, N-ethylmaleimide-sensitive fusion protein; and SNAP-25, synaptosomal-associated protein of 25 kDa.

The disease

Botulism can be divided into several different categories; classic or foodborne botulism, wound botulism, infant botulism, adult infectious botulism, inadvertent botulism and inhalation botulism.

Foodborne botulism is an intoxication caused by consuming food containing preformed neurotoxin. It is the most common cause of botulism in Europe. Many different types of food have been implicated, but the most common ones are home-made preserved foods. The type of toxin varies with location and type of food. For fish and seafood, type E is the most common, while in meats and vegetables types A and B dominate (Dodds, 1993a). The incubation period is usually 18-36 hours (Bell and Kyriakides, 2000). The first symptoms are often gastrointestinal, e.g. nausea, vomiting, stomach cramps, diarrhoea, or constipation. Many patients complain about having a dry mouth, muscle weakness and blurred or double vision (diplopia). Other symptoms are dysphagia (difficulty in swallowing), dysphonia (difficulty in speaking), dysarthria (slurred speech), ptosis (drooping eyelids), ophthalmoplegia (eye muscle paralysis), and a symmetric descending paralysis. It begins with the cranial nerves, followed by the upper extremities, the respiratory muscles, and the lower extremities (Shapiro et al., 1998).

Wound botulism occurs when a wound is contaminated with *C. botulinum* spores which germinate and produce toxin *in situ*. Wound botulism has been, except at times of war, a very rare form of botulism. It occurred mainly in patients with traumatic and surgical wounds, contaminated with dirt, e.g. shrapnel wounds. Between 1943 and 1990 only 47 cases were reported to the CDC and in literature (Cherington, 1998). Since then, however, there has been an upsurge of cases (Cherington, 1998; Jensen et al., 1998; Passaro et al., 1998; Jensenius et al., 2000; Brett et al., 2004; Akbulut et al., 2005; Alpers et al., 2005) (Paper I). The new risk group is injecting drug users, especially if they use skin or muscle “popping”. Soft tissue infections at injection sites can be caused by several different *Clostridium spp.* as well as by *Bacillus cereus* (Mitchell and Pons, 2001; Dancer et al., 2002). This may be due to dirty injecting paraphernalia or contaminated drugs. Drugs are often dissolved in acids and this enhances the breakdown of tissues and may accelerate bacterial growth. The symptoms are similar to those in classical botulism, but without gastrointestinal symptoms, and with an incubation period of 4-14 days (Shapiro et al., 1998). The symptoms may also appear in a different order than the classical picture (Brett et al., 2004).

Infant botulism occurs when *C. botulinum* colonises the intestines of infants younger than 12 months and produces toxin *in situ*. It is the most common cause of botulism in the United States, where 90% of the world's cases are diagnosed (Cox and Hinkle, 2002). It is believed that colonisation can occur since a normal bowel flora has not yet been established that could compete with *C. botulinum* (Sobel, 2005). The symptoms include constipation, lethargy, listlessness, general muscle weakness and “floppy head”, weak cry, poor sucking ability and difficulty in swallowing (Midura, 1996). There is a risk that the fulminant form, which leads to sudden and unexpected death, is mistaken for sudden infant death (Sonnabend et al., 1985; Bohnel et al., 2001; Nevas et al., 2005). Soil and honey are the two recognised sources of spores involved in infant botulism, but at least in one case infant formula milk powder has been implicated (Brett et al., 2005).

Adult infectious botulism, sometimes called hidden botulism, is similar to infant botulism in that the intestinal tract is colonised and the toxin produced *in situ*. It is unusual and occurs mainly in patients with an abnormality in the gastrointestinal tract, e.g. prior surgery, achlorhydria, Crohn's disease, or recent antibiotic treatment (Cherington, 1998).

It has long been held that the infectious forms of botulism (wound, infant, and hidden) can only be caused by type I strains, *C. butyricum*, and *C. baratii*, which all have optimal growth temperatures close to body temperature (Dodds, 1993c). However, we diagnosed a case of wound botulism caused by *C. botulinum* type E, a type II strain (Paper I).

Inadvertent, or iatrogenic, botulism has been caused by injection of botulinum toxin for cosmetic or therapeutic purposes. It seems to have occurred both in patients treated with doses below the maximum recommended dose (Cherington, 1998) and when unlicensed, highly concentrated toxin was used (Sobel, 2005; Chertow et al., 2006).

Inhalation botulism may result from aerosolisation of neurotoxin (Lindström and Korkeala, 2006a) and is not a naturally occurring disease (Sobel, 2005). It may occur in the laboratory setting when enough care is not taken or through the deliberate dissemination of the toxin (Arnon et al., 2001), e.g. as a weapon of bioterrorism.

Treatment of botulism consists mainly of advanced medical and nursing supportive care (Cherington, 1998). The mortality rate dropped sharply when mechanical ventilation was introduced in the 1940s and 1950s (Sobel, 2005). The only specific treatment for botulism is botulinum antitoxin. It can stop the progression of paralysis and decrease the time of hospitalisation. To give the best effect it should be administered early, preferable within 24 h of the onset of symptoms, since it only neutralises unbound toxin molecules (Sobel, 2005). However, effect has been seen as late as after four days (Chang and Ganguly, 2003). Since

this antitoxin is of equine origin there is a problem with adverse effects, including allergic reactions, anaphylactic shock, and serum sickness. This means that skin testing should be done before administration and that it can not be given to infants.

For infants, only supportive and respiratory care is given. The prognosis is still good, with a fatal outcome in less than 2% of the cases. Antibiotic treatment should not be given, as this may cause toxin to be released when the cells die and lyse. It may also destroy the normal microbial flora, helping overgrowth of *C. botulinum*. Some antibiotics, like the aminoglycosides, can even increase the neuromuscular weakness caused by *C. botulinum*, e.g. gentamicin may potentiate sublethal concentrations of toxin, resulting in complete neuromuscular blockade (Brook, 2007).

Recently an antitoxin of human immunoglobulin has been tested for the treatment of infant botulism (Arnon et al., 2006). The results seem promising, with a reduction in the length of hospital stay, but so far this antitoxin is only available in the US. Work is also being done to develop recombinant monoclonal antibodies as antitoxin, but this work is complicated by the existence of several subtypes within the serotypes (Smith et al., 2005). In the case of wound botulism suspected sites of infection are surgically debrided and intravenous benzylpenicillin combined with metronidazole given as well as antitoxin (Sieradzan, 2005).

Genetics

The *C. botulinum* genome has a very low content of d(G/C) DNA. The preferred initiation and termination sequences are AUG and UAA, and the use of degenerate codons ending in C or G is discriminated against. In the gene encoding neurotoxin type A, *cntA*, 90.3% of degenerate codons end in A or U (Thompson et al., 1990).

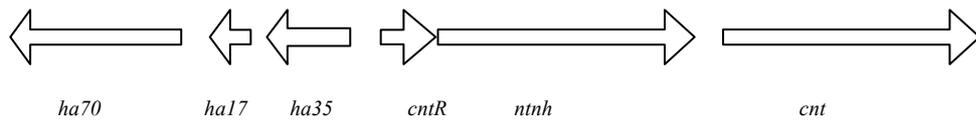
The nucleotide sequence of the neurotoxin gene has been identified for all toxin types, as well as some of the surrounding genes (Binz et al., 1990a; Binz et al., 1990b; Kimura et al., 1990; Thompson et al., 1990; East et al., 1992; Poulet et al., 1992; Whelan et al., 1992; Campbell et al., 1993). Recently the complete genome of three *C. botulinum* type A strains and one type F strain have been reported (accession CP000726, CP000727, AM412317, and CP000728), as well as the sequence of the plasmid pBOT3502 in ATCC 3502 (AM412318) and the type C neurotoxin-converting phage (AP008983).

The neurotoxin genes (*cnt*) in *C. botulinum* are located in a cluster with the genes coding for the ANTPs in the botulinum locus, and all the genes in the cluster are expressed together (Bradshaw et al., 2004; Couesnon et al., 2006) (Paper IV). The genes are transcribed as two divergent polycistronic messages, with the *ntnh* and *cnt* in one and *ha70*, *ha17* and *ha33* in the other. The organisation of the genes in the locus varies, as can be seen by the variation in the neurotoxin complex size. The 3' part tends to be more conserved than the 5' part (Raffestin et al., 2004). A gene called *botR* or *cntR*, also located in the locus, has been found in all types except non-proteolytic type E and acts as a positive regulator (Marvaud et al., 1998). The organisation of the genes in the different types can be seen in Figure 2. There is great genetic diversity between strains, even within the same type, and it has been suggested to further divide them into subgroups. For types A and B four subtypes each were found and for type E five subtypes (Hill et al., 2007).

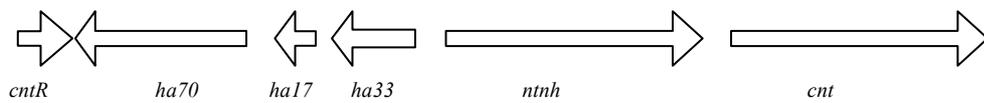
There is some confusion in literature about the names of the genes, since they have different designations in different articles. The neurotoxin gene itself was originally called *bont*, for botulinum neurotoxin, followed by type (i.e. *bontA*, *bontB*, etc). The designation *cnt*, for clostridial neurotoxin, followed by type (i.e. *cntA*, *cntB*, etc.) was suggested by the ASM Publications Board Nomenclature Committee (Lövenklev et al., 2004) (Paper II). Recently Sebahia et al. (Sebahia et al., 2007) suggested that all clostridial neurotoxins (including tetanus toxin) should be called *cntA*, presumably followed by toxin type. They also suggested that all non-toxin non-hemagglutinin genes, earlier known as *ntnh*, be called *cntB*. The hemagglutinin genes are known as *ha33*, *ha34* or *cntC*, *ha17* or *cntD*, and *ha70* or *cntE*. The

positive regulator is known as *p21*, *botR* or *cntR*. In type A2, E, and F, which lack hemagglutinin genes, three other open reading frames are found; *orfX1*, *orfX2*, and *p47* (East et al., 1996; Kubota et al., 1998). In this work the designations suggested by ASM will be used, except in Paper IV where the designations advocated by Sebahia et al. are used.

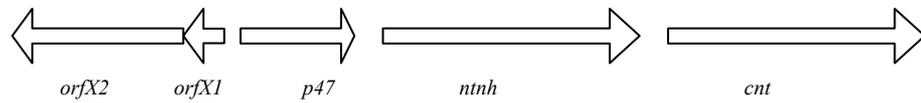
Types A1, B



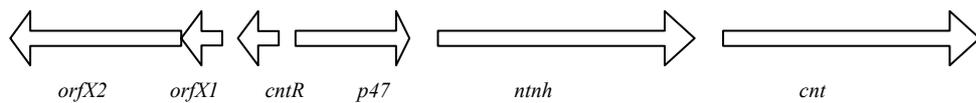
Types C, D



Type E



Types A2, F



Type G

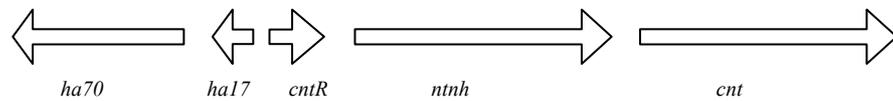


Figure 2 Organisation of the genes in the neurotoxin gene cluster. Adapted from Lövenklev (Lövenklev, 2003).

Diagnostics and detection methods

Preliminary diagnosis of botulism is based on clinical symptoms and patient history. It is probably an underdiagnosed disease, and may be misdiagnosed as myasthenia gravis, Guillain-Barré syndrome, stroke, tick paralysis, chemical intoxication, chemical or bacterial food poisoning, mushroom poisoning, medication reactions, poliomyelitis, diphtheria, or psychiatric illness. When infants are involved, meningitis, electrolyte-mineral imbalance, metabolic encephalopathy, congenital myopathy, Reye syndrome, Werdnig-Hoffman disease, and Leigh disease may also be suspected (CDC, 1998). Botulism cannot be diagnosed using routine laboratory tests, however examination of the cerebrospinal fluid (CSF) and electromyography (EMG) can help rule out other diseases. To confirm the diagnosis, identification of the toxin in samples like serum, vomitus, gastric aspirate, stool, or culture thereof is necessary.

The mouse bioassay is currently the only internationally accepted method of confirming BoNTs. Suspect material is intraperitoneally injected into mice which are monitored for botulism symptoms. The toxin type is determined using mice protected with monovalent, serotype-specific antibodies. If possible the organism is isolated. However, for food botulism laboratory tests have been reported to be positive in only 65% of the cases (CDC, 1998) and for wound botulism in the corresponding figure is only around 50% (Sieradzan, 2005). One reason for this may be that several of the commercially available test systems for identification of anaerobic bacteria have a problem with correctly identifying *C. botulinum* (Lindström et al., 1999). While the mouse bioassay is very sensitive, it has several drawbacks. It is time and labour expensive, requires a special animal facility and trained personnel, and can take up to four days. This, as well as the ethical issues of using animal testing, make the method unsuitable for large-scale testing and screening. This has led to the development of several new ways of detecting the organism and its toxin. Most toxin detection methods are based on immunological reactions, such as enzyme-linked immunosorbent assay (ELISA) (Ferreira et al., 2003), enzyme-linked coagulation assay (ELCA) (Doellgast et al., 1993), lateral flow tests (Sharma et al., 2005; Gessler et al., 2007), immuno-PCR (Wu et al., 2001), electrochemiluminescence (ECL) (Rivera et al., 2006), time-resolved fluorescence assay (Peruski et al., 2002), and biosensors (Ogert et al., 1992). Some detect the activity of the toxin, like the endopeptidase activity assay (Hallis et al., 1996; Wictome et al., 1999), endopep-MS (Kalb et al., 2006), and the neuron-based cellular assay (Dong et al., 2004; Sheridan et al., 2005). A review of the advantages and disadvantages of each was recently published by Cai et

al. (Cai et al., 2007). The main problem in testing for the toxin is that only unbound toxin can be detected. When testing for the organism, culture-based or nucleic acid-based methods, or combinations thereof are used. The nucleic acid-based methods include PCR, both conventional (Szabo et al., 1993; Franciosa et al., 1994; Fach et al., 1995; Hielm et al., 1996; Takeshi et al., 1996; Aranda et al., 1997; Alsallami and Kotlowski, 2001; Dahlenborg et al., 2001; Lindström et al., 2001; Chaffer et al., 2006) and real-time (Kimura et al., 2001; Akbulut et al., 2004; Yoon et al., 2005; Christensen et al., 2006; Fenicia et al., 2007), and DNA arrays (Song et al., 2006). Lindström et al. (2006a) have recently reviewed both culture methods, immunological methods, typing methods, and PCR protocols and compared their sensitivities, reproducibility, and ease of use. They found that a combination of methods is often needed, as each method has different strengths and weaknesses.

Diagnostic PCR

In the 20 years since the polymerase chain reaction (PCR) was first described (Mullis and Faloona, 1987), this method of selectively amplifying a DNA segment has become an increasingly important tool in microbial diagnostics. Its main advantages are that it is very rapid, specific, and can detect organisms that are hard or impossible to detect by culture (Sachse, 2003). When it comes to viruses, an improved sensitivity is observed in almost all instances when compared to culture (Cockerill, 2003; Niesters, 2004). In addition to being very specific, PCR can be used to selectively determine things like subtype, genotype, variant, mutant, and genotypic resistance patterns (Niesters, 2004). Today, diagnostic PCR is used in many fields, such as detection of genetically modified organisms (GMO), forensics, food microbiology, epidemiology, and detection of genetic diseases.

Real-time PCR

While conventional PCR requires post-amplification detection, like agarose or polyacrylamide gel electrophoresis, Southern blotting, sequencing, or ELISA (Neumaier et al., 1998), real-time PCR continually measures the accumulation of amplicon using fluorescence. This is done using either a dye that binds to all double-stranded DNA, like ethidium bromide (Higuchi et al., 1992) or SYBR Green I (Wittwer et al., 1997), or by labelling of primers (Chehab and Kan, 1989) or probes with fluorogenic compounds. There are several different types of probes, most of which use fluorescence resonance energy transfer (FRET). FRET is a spectroscopic process where energy is passed non-radiatively between molecules through dipole-dipole resonance transfer (Clegg, 1992). The fluorophore donor molecule absorbs a photon and transfers the energy to an acceptor molecule. The acceptor may be a quencher, which does not fluoresce, as is the case with hydrolysis probes (Holland et al., 1991) and molecular beacons (Tyagi and Kramer, 1996), or another fluorophore which emits light at a different wavelength, like in hybridisation probes (Wittwer et al., 1997). The distance between the donor and the acceptor molecule can be of macromolecular dimensions (10-100 Å), but the efficiency of the transfer is affected by the distance (Clegg, 1992).

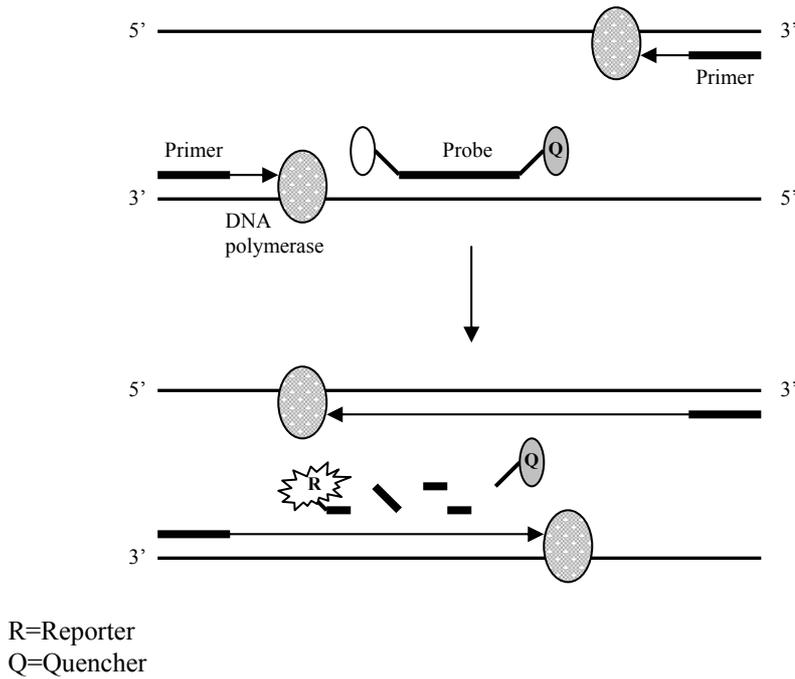
Besides not needing post-amplification detection and thereby reducing the risk for carry-over or cross-contamination, real-time PCR has the advantage of being quantitative. This is because what is measured is not how much product you have at the end, when the reaction might have reached a plateau phase, but at what cycle (Ct or Cp depending on what type of instrument is used) the product reaches a detectable amount during the exponential increase of PCR product. This means it is measured in the early logarithmic stage of the amplification (Higuchi et al., 1993). There are many cases where this quantification is very useful. In virology, quantitative PCR can help determine the viral load and if intervention with pre-emptive antiviral strategies is required (Niesters, 2004), or to monitor the progression or eradication of infection (Monpoeho et al., 2002). It can be used to quantify the bacterial or viral load in food samples (Kimura et al., 2001; Wolffs et al., 2004b; Shan et al., 2005; Wolffs et al., 2005; Yoon et al., 2005) or to follow the expression of different genes (Mathews et al., 1999; Bustin, 2000; Jung et al., 2000; Schmittgen and Zakrajsek, 2000; Giulietti et al., 2001; Goerke et al., 2001; Rajeevan et al., 2001; Walker, 2001; Eleaume and Jabbouri, 2004; Lövenklev et al., 2004; Sharkey et al., 2004; Couesnon et al., 2006; Kouguchi et al., 2006; Shin et al., 2006).

When designing your assay there are many things to think about. The design of primers and probes is very important, as different primers can have 1000-fold different sensitivity for the same gene (Sachse, 2003) and they are the most important factor for deciding the selectivity of the assay. If there are primer-dimers generated this will decrease the sensitivity of the assay since it will be a competitive reaction and if SYBR green is used it will also make it difficult to separate the reactions, even though melting curve analysis can help. The PCR platform used can, as well as the type of probe and dye, influence the detection (Reynisson et al., 2006). The choice of probe type partially depends on the type of real-time PCR instrument used as some probe chemistries are more suited to particular instruments. This is especially true if multiplexing is desired, since the wavelengths emitted and detected determine how many and which dyes can be used. When multiplexing it is also important to make sure the colours can be clearly separated, not spilling over into each other's channels.

Once primers and probes are chosen the rest of the reaction can be optimised. The usual parameters that need attention are primer-, probe-, and magnesium concentrations and annealing temperature. While the amounts of dNTPs and DNA polymerase are usually standard, the type of DNA polymerase can play an important part in determining not just the amplification efficiency and detection window (Wolffs et al., 2004a), but also the robustness of the assay, since inhibition affects different DNA polymerases in different ways (Abu Al-Soud and Rådström, 1998; Löfström et al., 2004). What is optimal for one type of sample may not always be the best for another type. In some cases adding a facilitator may help overcome inhibitions (Abu Al-Soud and Rådström, 2000).

In this work, two different probe chemistries have been used. In Papers II and IV TaqMan probes (Livak et al., 1995) were used, while in Papers I and III hybridisation probes (Wittwer et al., 1997) were used. The principles of the two systems are described in Figure 3.

A *TaqMan* probe



B Hybridization probe

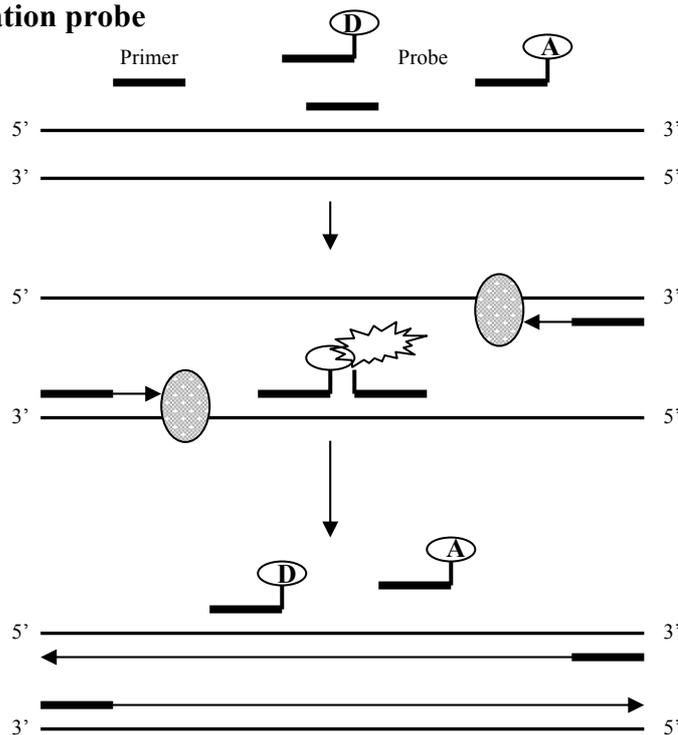


Figure 3 Principles of TaqMan probes (A) and hybridisation probes (B).

A. After hybridising to the template during the annealing step, the probe is degraded by the exonuclease activity during the extension step. When the reporter dye (R) is separated from the quencher (Q), a fluorescent signal can be measured.

B. When the two hybridisation probes hybridise close to each other, the energy from the donor probe (D) is transferred to the acceptor probe (A) through FRET, and an increase in the fluorescent signal can be measured. Used with permission of Maria Lövenklev (Lövenklev, 2003).

Internal amplification control

Many clinical and food samples contain substances which can inhibit PCR. They may do this by degrading the DNA polymerase, by denaturing it, or simply reducing the activity e.g. by binding the magnesium ions needed by the enzyme. Other inhibitors may degrade or capture the nucleic acids or, when using real-time PCR, interfere with the fluorescence. The pre-PCR processing approach used should try to eliminate these inhibitors (Rådström et al., 2004) and the use of an alternative thermostable DNA polymerase (Abu Al-Soud et al., 1998; Wolffs et al., 2004a) and amplification facilitators (Abu Al-Soud et al., 2000) can help further. The most used methods of avoiding inhibition are physical methods, like centrifugation and filtration, DNA extraction methods, and adsorption (Lantz et al., 2000). But even using all these methods there is still a risk that there will be inhibitors present in the sample that may cause a false-negative result. In order to avoid this, an internal amplification control (IAC), which is present in every tube, needs to be added to the assay. When an international standard for PCR-based detection of pathogenic microorganisms in food is being created, inclusion of an IAC has become mandatory (EN ISO 22174).

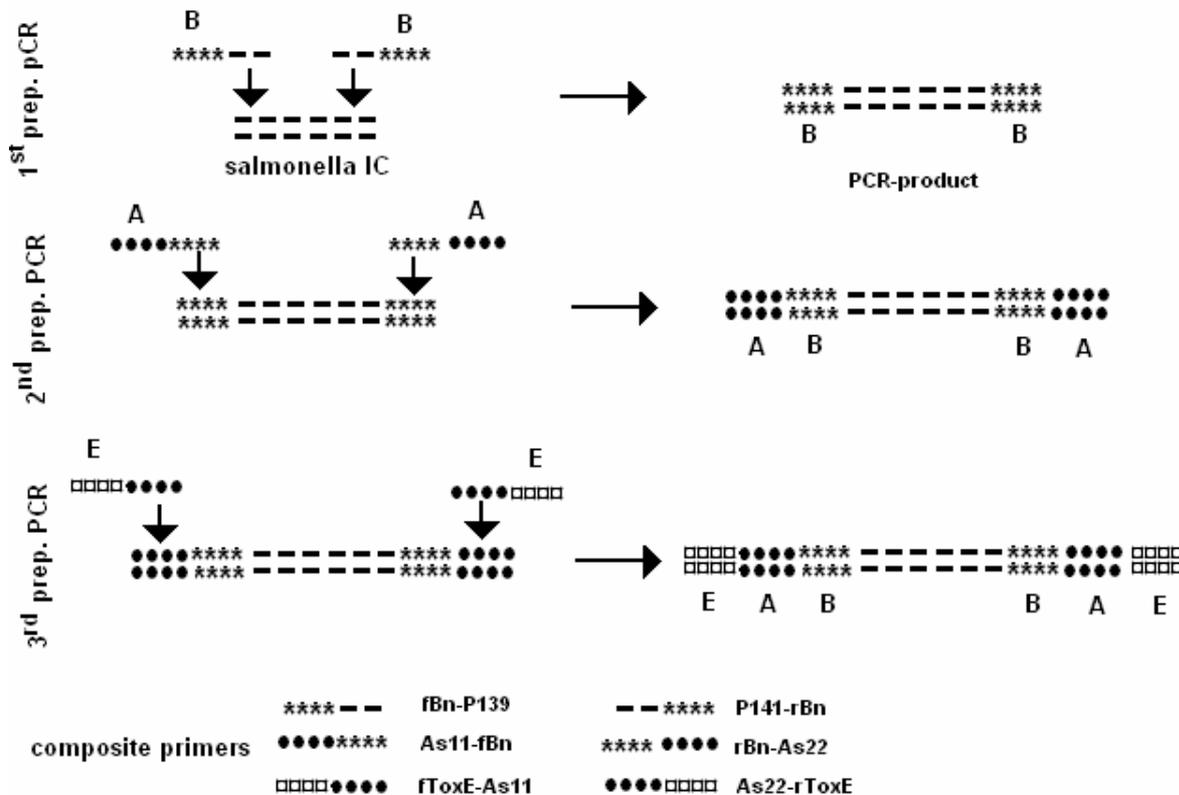


Figure 4 A schematic picture of the generation of the multiple internal amplification control.

The two main types of IACs are competitive, which uses the same primers as the target, and non-competitive, which uses different primers (Hoorfar et al., 2004). We used the method described by Stöcher et al. (2002) to generate a multiple competitive IAC for the diagnostic assays used in this thesis (Paper I). Using an existing internal control for *Salmonella* spp (IC T69), which contained a binding site for a pair of hybridisation probes (Perelle et al., 2004), as starting material, additional primer-specific sequences were added in conventional preparative PCR with composite primers (Figure 4). This was done in such a way that the amplicon from the IAC would always be larger than the amplicon from the target, since the amplification of the smaller target is favoured by the reaction kinetics (Toouli et al., 2000; Sachse, 2003; Hoorfar et al., 2004), and to be able to visually discriminate the products on a gel. Since the internal controls are used as competitors with the same primers as the clinical targets the amount of internal control present in a sample needs to be carefully titrated in order to avoid reduced amplification efficiency (Hoorfar et al., 2004). The optimal amount of IAC in the competitive PCRs was determined by comparing target detection with and without the presence of different amounts of IAC (Figure 5). While it is important to keep the amount as low as possible in order to minimise the influence on the target detection, it must be high enough to give a reproducible detection of the IAC (Rodriguez-Lazaro et al., 2004) in the samples negative for bacterial DNA as well as in the most diluted ones. We found that the amount of IAC added in our assay did not significantly change the detection limit.

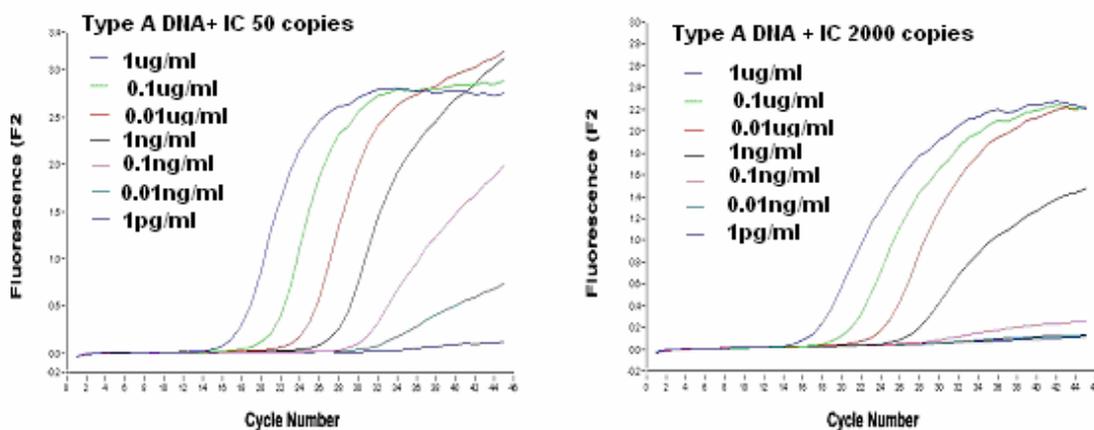


Figure 5 Effect of amount of internal control on detection of *C. botulinum* type A. When too much internal control is added the lowest concentration of target may not be detected.

Detection limits

While it is true that PCR in theory can detect as little as one DNA molecule, in practice there are several difficulties. The first one arises from the difficulty of getting that one molecule, in a sample of millilitres, or sometimes even more, into the few microlitres that are added to the reaction. Sheer probability tells us that even if there is a theoretical concentration of one molecule per volume added, not every volume taken will contain a molecule. Therefore, instead of talking about a limit of detection it is more reasonable to talk about a detection probability at a certain concentration. Thus, while the lowest amounts of DNA that could be detected for *C. botulinum* types A and E were equivalent to 0.5 and 1 genome copy respectively, the lowest concentrations that could be reliably detected corresponded to 5 and 25 copies respectively (Paper I). The detection probabilities at different DNA concentrations can be seen in Figure 2 of Paper I.

Selectivity

The selectivity of the diagnostic PCR assays were evaluated using 40 strains of *C. botulinum*, 16 strains of other *Clostridium* spp., and 26 non-*Clostridium* strains (Paper I). The selectivity is a measure of the inclusivity, i.e. the ability to detect different strains of the target pathogen, as well as the exclusivity, i.e. not detecting anything else (Malorny et al., 2003). The selectivity was found to be good, with amplification of all the correct strains, no amplification of other strains, and no cross-reaction between strains.

The problem when designing the assay is that the number of known sequences is limited, though increasing rapidly, and if the target sequence is not highly conserved there is a risk that some strains may contain variations not previously reported (Paper I). If these variations are in the area where the primers or probes are located a false-negative may be the result. This problem grows when the exclusivity is increased. While probes increase the specificity of the assay by increasing the number of places where DNA must match, this also means that there are more places where a variation can result in non-detection. This means that it is important to keep evaluating the assay as more and more strains are available for testing and more sequences are reported to sequence databases.

Types of samples

In the cell genetic information is stored as DNA. When protein production is needed the DNA is transcribed into mRNA which is then translated into polypeptides, which become the protein, after suitable post-translational modification. This gives us three different possibilities of monitoring the bacteria (Figure 6). By determining the presence of a specific gene, e.g. the toxin gene, we determine if toxigenic bacteria are present in the sample (Paper I). By measuring mRNA levels we can decide if the genes are active and determine the level of activity (Papers II, III, and IV). Finally, by measuring the concentration or activity of the toxin we can determine if the protein is being produced and released (Papers I, II, III, and IV). This can be done using *in vitro* methods like ELISA (Ferreira et al., 2003) or *in vivo* methods like the mouse bioassay (Holdeman et al., 1977).

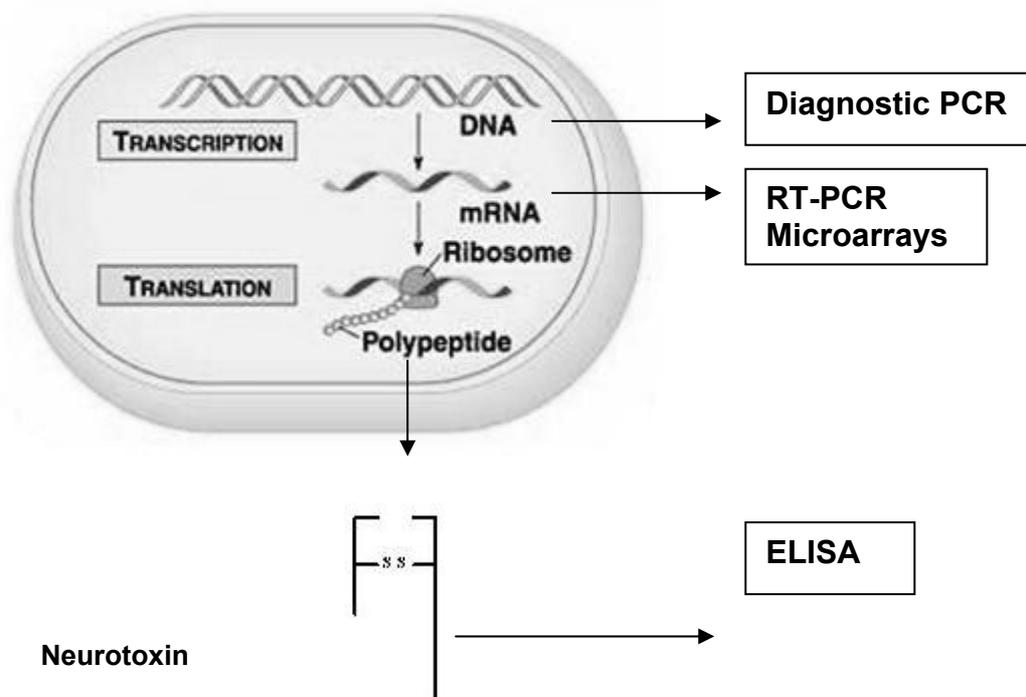


Figure 6 Different points at which samples can be taken.

Neurotoxin expression during growth

Reverse transcription PCR (RT-PCR)

For a long time molecular biologists were convinced that the flow of genetic information is unidirectional, from DNA to RNA to protein (Culliton, 1971). However, in 1964 it was found that cells infected with Rous sarcoma virus, an RNA virus, contained new DNA homologous to the viral RNA (Temin, 1964). Six years later, two independent groups found evidence of the existence of an enzyme which can synthesise DNA from an RNA template (Baltimore, 1970; Temin and Mizutani, 1970). In 1971 the RNA-dependent DNA polymerase, or reverse transcriptase as it is now known, from the avian myeloblastosis virus was purified and characterised (Kacian et al., 1971) and it was shown that it would accept genetically unrelated RNA molecules as a template (Spiegelman et al., 1971). This meant that the same enzyme could be used to reverse transcribe many different types of RNA, which could in turn be used as a template for PCR (Rappolee et al., 1989). It is possible to do this in a one-step protocol, where the reverse transcription and PCR amplification takes place in the same tube. This has, however, been seen to be less sensitive than using a two-step procedure (Bustin, 2002). An oligonucleotide primer is required to initiate the synthesis of the complementary DNA (cDNA). The first ones described were oligo(dT) primers (Rappolee et al., 1988), which will reverse transcribe all messenger RNA (mRNA) from eukaryotes. This approach is not possible when working with prokaryotes as they do not produce mRNA with a polyA-tail. The other alternatives are random hexadeoxynucleotide primers (Noonan and Roninson, 1988), which will also reverse transcribe all RNA, and specific primers (Chelly et al., 1988), which will only reverse transcribe RNA from the gene of interest. The advantages and disadvantages of each were recently reviewed by Bustin et al. (Bustin et al., 2005). In 1989 it was shown that reverse transcription PCR (RT-PCR) could be used to quantify specific mRNA sequences in a reproducible way (Becker-Andre and Hahlbrock, 1989; Delidow et al., 1989).

In the present work quantitative RT-PCR (qRT-PCR) methods were developed to monitor the *cntB* expression in *C. botulinum* type B (Paper II), *cntE* expression in *C. botulinum* type E (Paper III) and *cntA* and *cntR* expression in *C. botulinum* type A (Paper IV). We use a two-step procedure where the mRNA is first reverse transcribed using Superscript II and specific primers, then quantified with real-time PCR using *Tth* polymerase and probes. This system

was developed after comparing several different methods, both one-step and two-step, and enzymes.

Real-time PCR quantification

As mentioned earlier, real-time PCR is quantitative. There are several different ways this can be used. Absolute quantification can be performed by comparing Ct values to a standard curve constructed with known concentrations of target. This is more useful when quantifying DNA than mRNA, since a pre-constructed standard does not take differences in RT efficiency into account. While it would be possible to include RNA standards in the RT step, insuring the same transcription efficiency, there is a problem with the stability of the standards, as RNA tends to degrade faster than DNA. This means that the method is generally only used when absolute quantification is essential, like in quantifying viral loads. The principle behind a standard curve can be seen in Figure 7.

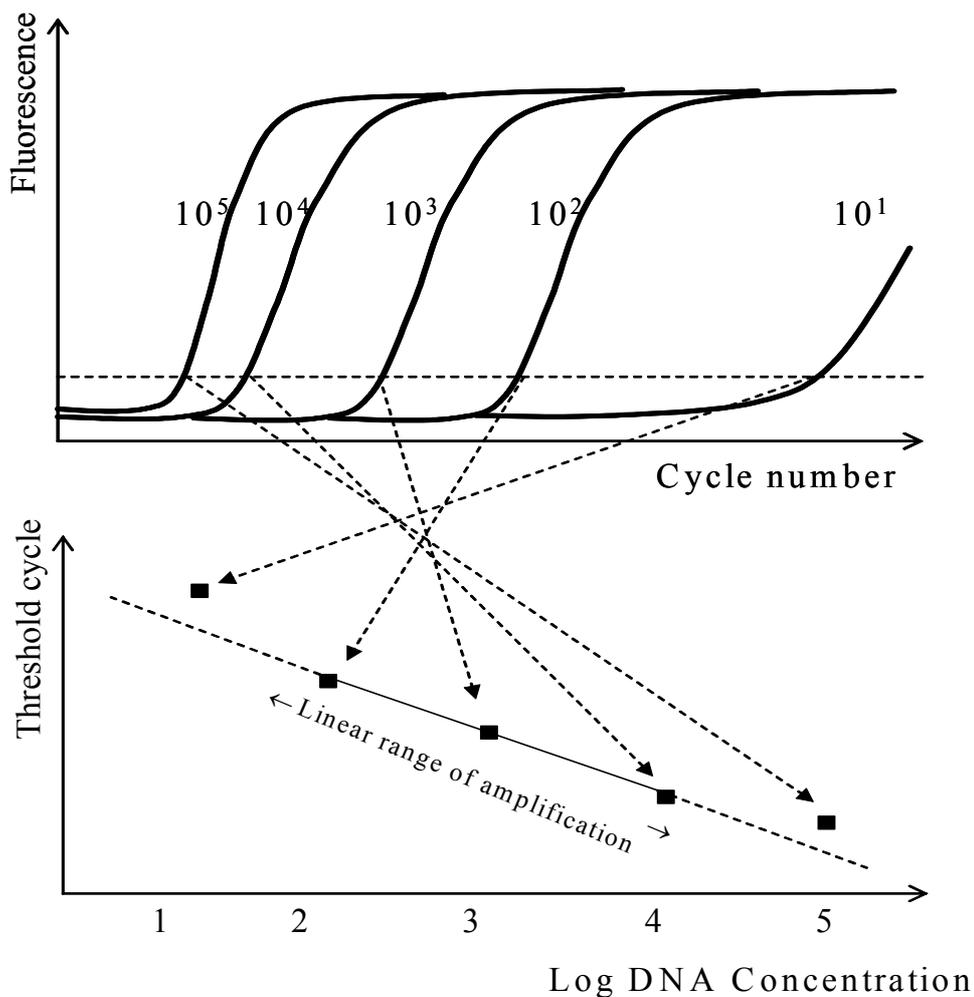


Figure 7 Schematic picture of standard curve. While product can be detected in the entire detection window, quantification assuming linear relationship is only valid in the linear range of amplification. Used with permission of Petra Wolffs (Wolffs, 2004).

Relative quantification can be performed by correcting for differences in RNA input or reverse transcription efficiencies by normalising them to a housekeeping gene. This should be a gene that is expressed at a constant level in all the relevant times and conditions tested (Giulietti et al., 2001). Another method for relative quantification is the comparative Ct method. In the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) the amount of target relative to a calibrator and normalised to a housekeeping (reference) gene is given by

$$\text{amount of target} = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{calibrator})$$

$$\Delta Ct = Ct(\text{target}) - Ct(\text{reference})$$

The biggest problem with this method is that it requires close to identical amplification efficiencies of target and housekeeping gene. While efficiencies close to 100% should always be sought, it is not always possible to achieve this. Pfaffl (Pfaffl, 2001) solved this problem by including the amplification efficiency in his formula for calculating relative expression (RE)

$$RE = \frac{((1+E_{target})^{\Delta Ct_{target}(\text{calibrator- sample})})}{((1+E_{reference})^{\Delta Ct_{reference}(\text{calibrator- sample})})}$$

The amplification efficiency (E) is

$$E = (10^{(-1/s)}) - 1$$

where s is the slope of the log-linear range of amplification (Figure 7) (Klein et al., 1999).

Microarrays

A new method of gene expression profiling is beginning to play a bigger part as more and more genomes are being sequenced. DNA microarrays, membranes or glass slides with a mosaic of the entire genome, either as oligonucleotides (oligonucleotide microarrays) or as PCR products representing the individual genes (cDNA microarrays), give the possibility to assess transcription on a genomic scale. The two technological advances mainly responsible for the development of microarrays are application of robotic technology to routine molecular biology and dual fluorescent labelling (Lucchini et al., 2001). In addition to being used to examine expression microarrays can be used for genome typing and to study microbial evolution. A microarray based on the genome of the *C. botulinum* type A strain ATCC 3502 has recently been used for comparative genomic indexing (Sebahia et al., 2007). This is the same type of microarray used in Paper IV. Figure 8 illustrates the placement of the array probes in the toxin gene cluster.

Briefly, reference and sample DNA or cDNA is labelled, one with Cy3, the other with Cy5. They are then simultaneously hybridised to the microarray slide, which thus is unaffected by variability in hybridisation or differences between individual microarrays (Lucchini et al., 2001). After hybridisation the ratio between the amount of reference and sample that has hybridised can be read with a microarray scanner and the digital image can be processed. Grids specifying the location of the target genes can be overlaid on the images (Duggan et al., 1999). Either DNA or cDNA can be used as a reference. In this work genomic DNA was used as a reference (Paper IV). Labelling the mRNA can either be done by first reverse transcribing it, then labelling it the same way the reference DNA is labelled, or by directly labelling it during the reverse transcription. We chose the direct labelling approach, in order to minimise the effect of any remaining DNA in the RNA preparation (Paper IV). An overview of the setup can be seen in Figure 9.

By studying the transcriptome, i.e. changes of gene expression and transcription on a global scale, a vivid picture of the cell's adaptation to a changing environment can be seen. Furthermore, the behaviour of large groups of functionally related genes can be studied and common regulatory functions found for sets of genes which show similar expression profiles (DeRisi et al., 1997).

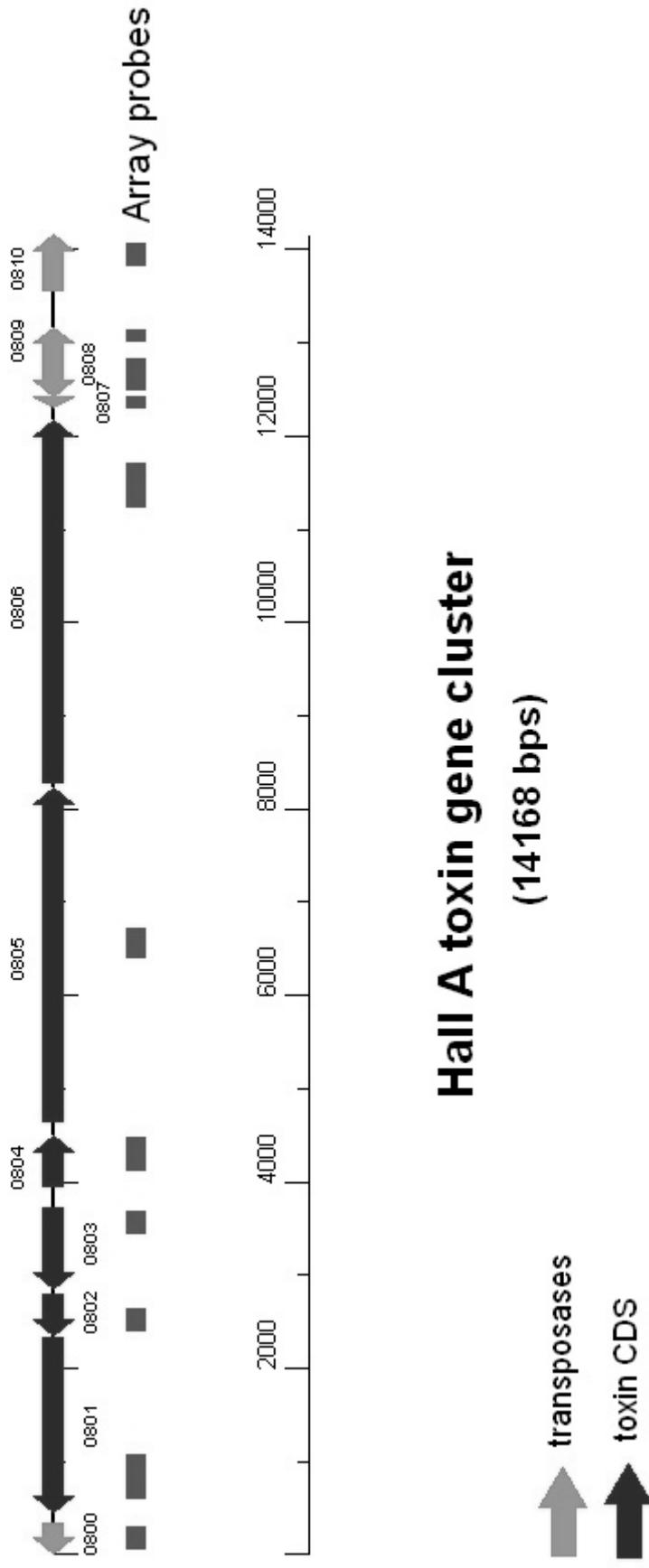


Figure 8 Organisation of genes and microarray probes of the neurotoxin gene cluster in ATCC 3502. The numbering of the coding sequences is according to Sebahia et al. (Sebahia et al., 2007). CBO0800 - putative transposase (partial), CBO0801- hemagglutinin component of the neurotoxin complex, CBO0802 - hemagglutinin component of the neurotoxin complex, CBO0803 - hemagglutinin component of the neurotoxin complex, CBO0804 - component of the neurotoxin complex (cntR), CBO0805 - type A progenitor toxin nontoxic-nonhemagglutinin, CBO0806 - botulinum neurotoxin type A precursor, CBO0807 - transposase (pseudogene), CBO0808 - transposase/integrase, CBO0809 - putative transposase (pseudogene), CBO0810 - putative transposase for insertion sequence element IS904. Used with permission of Andrew Carter (IFR, Norwich, UK).

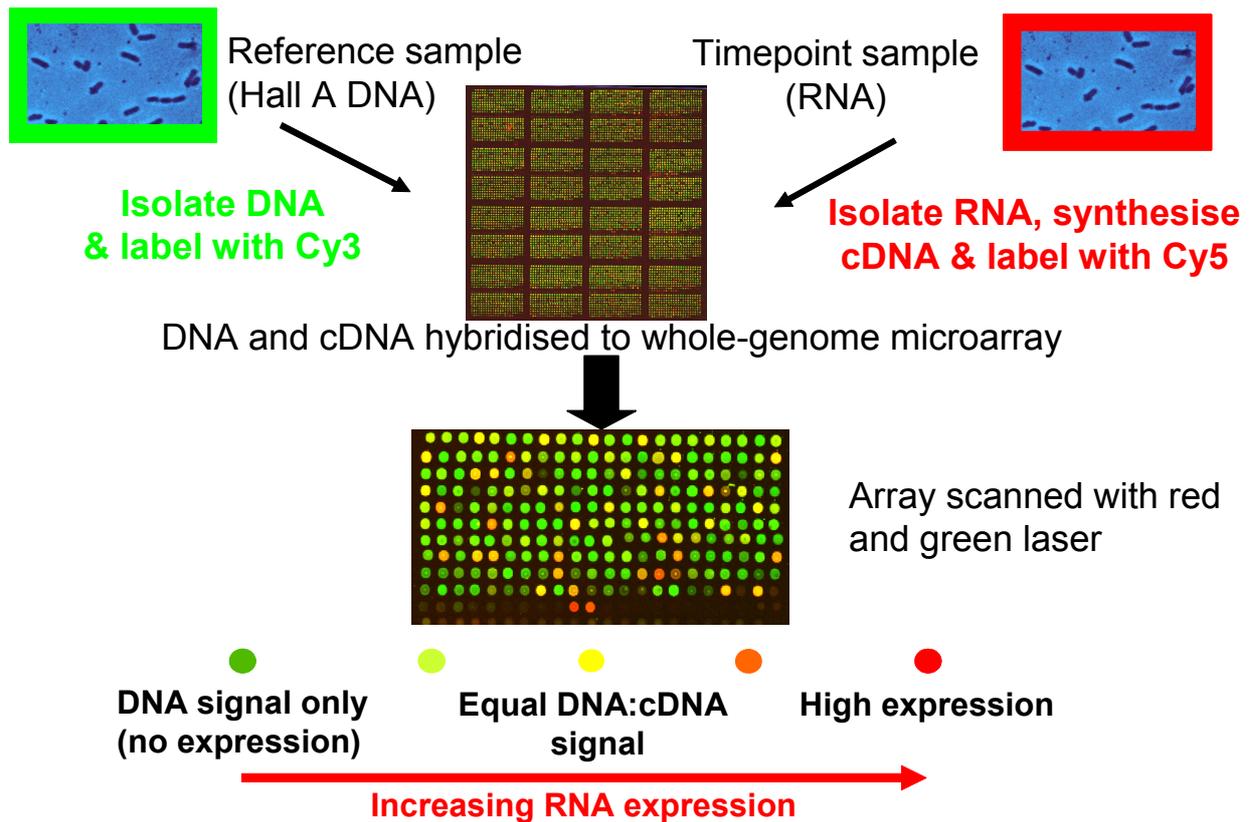


Figure 9 Setup for Hall A microarray analysis. The reference DNA is labelled with Cy3 and the sample cDNA with Cy5. Each array contains approximately 3500 gene probes spotted in duplicate, arranged in 16 blocks. Each slide bears two arrays. Used with permission of Mike Peck and Andrew Carter (IFR, Norwich, UK).

Neurotoxin gene expression profiles

Little is known about the direct regulation of the neurotoxin gene (*cnt*), as most studies have focused on germination, growth and time to toxicity, using the mouse bioassay. However, knowing when a gene is expressed is often a strong indicator of its biological role. While regulation of mRNA is not the only way abundance of protein is regulated, most differences in cell types or states are correlated to changes in mRNA levels of several genes. A positive regulator, *cntR*, has been found in all *C. botulinum* types except type E. In this work we have followed the expression profiles of *C. botulinum* types A, B, and E during growth in different conditions (Papers II, III, and IV). In the case of *C. botulinum* type A we also followed the expression profile of *cntR* and the genome-wide expression using microarrays (Paper IV).

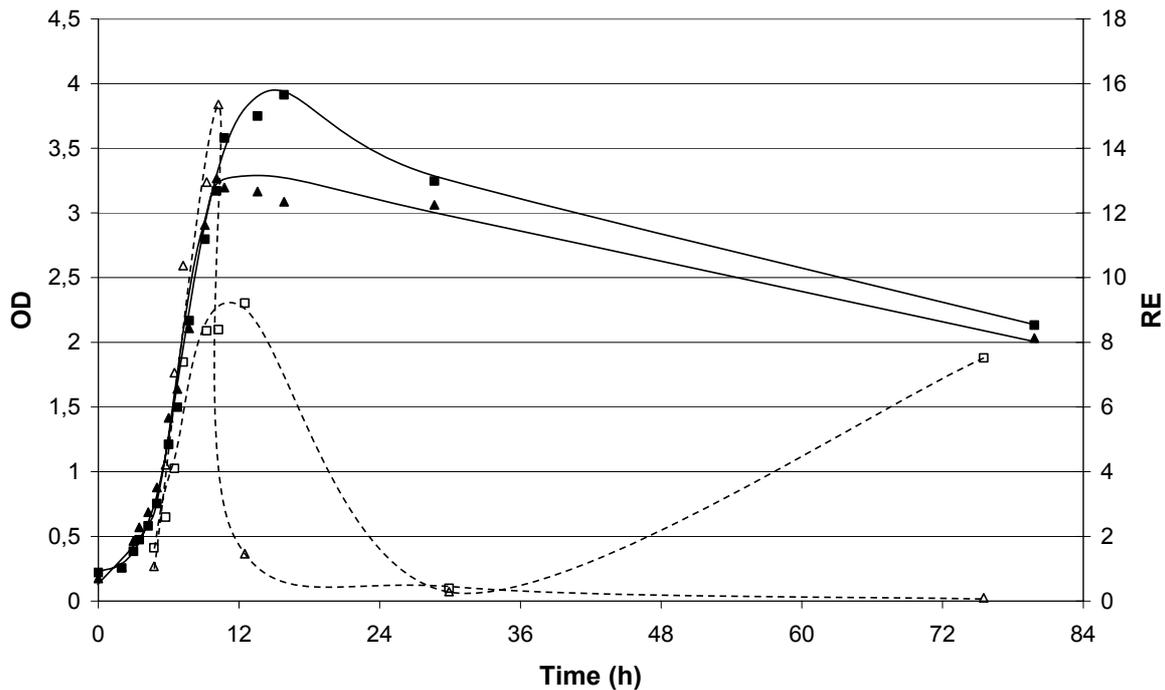


Figure 10 Growth and expression in *C. botulinum*. Squares – proteolytic type A (Hall A), triangles – non-proteolytic type E (ATCC 9564), whole lines – OD₆₂₀, dashed lines – relative neurotoxin expression.

When looking at the profiles of neurotoxin expression during the different growth stages it is easy to see some similarities between the different neurotoxin types of *C. botulinum*, as well as certain differences. They all seem to have a maximum relative *cnt* mRNA level during late exponential or early stationary phase and then to decline, but while type E goes down to a very low level and then stays there (Figure 10), some of the other types show secondary mRNA peaks in later phases. In some cases these peaks seem as high as or possibly even higher than the first peak. This behaviour seems more marked in proteolytic than in non-proteolytic strains. In the proteolytic type B strain ATCC 17841, Lövenklev et al. (Lövenklev et al., 2004) seem to see two later peaks, one, higher than the first one, at 11 h and one, approximately the same as the first one, at 30 h, while the other strains only showed the one at 30 h. The levels of expression differ between the different strains, with proteolytic strains generally showing higher levels. The highest expression levels and the highest concentrations of toxin were found in the proteolytic strain ATCC 7949. In proteolytic *C. botulinum* type A strain Hall A there was clearly a second peak in late stationary or death phase. It was in the same range as the first peak, and may have continued to rise.

Table 2 Genes with expression similar ($P \geq 0.99$) to neurotoxin gene cluster.

| Locus | P-value | Protein |
|----------------|----------------|---|
| CBO0026 | 0.996 | Membrane protein |
| CBO0433 | 0.990 | Hydrogenase nickel incorporation protein |
| CBO0575 | 0.990 | Two-component response regulator |
| CBO0644 | 0.990 | Conserved hypothetical protein |
| CBO0909 | 0.995 | GnaT-family acetyltransferase |
| CBO1134 | 0.990 | Pyrazinamidase/nicotinamidase |
| CBO1238 | 0.996 | Conserved hypothetical protein |
| CBO1254 | 0.997 | Thioredoxin reductase |
| CBO1288 | 0.995 | Guanine deaminase |
| CBO1402 | 0.997 | ABC transport system, ATP-binding protein |
| CBO1815 | 0.991 | Conserved hypothetical protein |
| CBO1848 | 0.992 | Electron-transferring subunit of iron-only hydrogenase |
| CBO1849 | 0.993 | Electron-transferring subunit of iron-only hydrogenase |
| CBO2021 | 0.990 | Chloramphenicol acetyltransferase |
| CBO2079 | 0.997 | Transmembrane protein |
| CBO2264 | 0.991 | Transporter protein |
| CBO2316 | 0.994 | Membrane protein |
| CBO2473 | 0.992 | Integral membrane protein |
| CBO2948 | 0.999 | Bifunctional membrane-associated kinase and phosphatase |
| CBO3515 | 0.995 | Translation elongation factor G, Ef-G |

The peak in late exponential phase has been seen in several strains of *C. botulinum* type A (Bradshaw et al., 2004; Couesnon et al., 2006; Shin et al., 2006), with strain KNIH-1 as the only exception (Shin et al., 2006), but the second peak is only mentioned as a “slight transient increase” by Couesnon et al. (2006) and not at all by the others. It may be that those strains did not have such a marked second peak, or that they did not follow the expression for a long enough period to detect it. It may also be an effect of the different medias used, as this is known to influence expression (Bradshaw et al., 2004). In Hall A the positive regulator gene *cntR* was also studied with real-time PCR. The expression profile was, as expected, very similar to the one of the gene complex it regulates, even if it was expressed at much lower levels.

When studying the genome-wide gene expression in Hall A with microarrays we found that the entire neurotoxin gene cluster is expressed as a whole, with similar expression levels, except for the positive regulator gene, *cntR*, which is expressed at a much lower level, CBO0800 (transposase), which is expressed at a higher level, and CBO0803 (HA34), which does not seem to follow any pattern at all (Paper IV, Figure 4). The latter may be because of problems with the probe, since Couesnon et al. (Couesnon et al., 2006) claim it follows the same pattern as the rest of the cluster, but at higher levels. There are several other genes that follow the same expression pattern as the toxin gene cluster (Table 2). Other genes are obviously different, many showing little variation in expression throughout the growth curve. Most of the other genes follow the predicted pattern, such as activation of the sporulation genes in the later parts of the growth curve (Paper IV, Figure 10).

Effect of CO₂

Modified atmosphere packaging

During the last decades modified atmosphere packaging (MAP) of food has become increasingly common. The main reason for this has been consumer demand for fresh and chilled products, preferably without preservatives, which will still keep for a long time. From the industry's side the extended shelf life means economic gains since packaging can be centralised and new types of products, like refrigerated ready-to-eat foods, can take the place of the declining canned and frozen foods (Farber, 1991). MAP is used for many different types of food, like fruits, vegetables, eggs, meat, bakery products, and seafood, as well as for ready-to-eat foods.

The three gases most used in MAP are oxygen (O₂), nitrogen (N₂), and carbon dioxide (CO₂). Oxygen is used mainly in MAP of meats to maintain the bright red colour and to prevent the growth of anaerobic bacteria (Farber, 1991). While *C. botulinum* is considered an anaerobic organism, spores can germinate in $\leq 1\%$ O₂ and vegetative cells can grow in 15% O₂ (Whiting and Naftulin, 1992), showing that inclusion of oxygen is not enough in itself.

Nitrogen is used as a filler gas and to displace oxygen, in order to delay oxidative rancidity and growth of aerobic spoilage bacteria. It is not antimicrobial in itself and it has been shown that it will allow *C. botulinum* to grow, while it retards spoilage (Kautter et al., 1981).

Carbon dioxide is the gas responsible for most of the retardation of microbial growth. It is most effective for foods whose spoilage flora consists of gram-negative, aerobic, psychrotrophic bacteria (Daniels et al., 1985). The mechanism for its effect is not completely understood and several theories have been suggested. While reducing the available oxygen by displacing it would interfere with aerobic organisms, CO₂ also affects anaerobes, and replacing it with 100% N₂ does not have the same effect, indicating that this is not the main reason for its effect. Other theories include an alteration of the cell membrane, leading to decreases in glucose uptake rates and amino acid absorption, inhibition of enzymes, or induction or repression of their production, penetration of the cell membrane, leading to intracellular pH changes, and direct changes to proteins, like their solubility, reactivity, stability, charge, or configuration (Daniels et al., 1985; Dixon and Kell, 1989). It is generally agreed that it is the CO₂ dissolved in the fat and water phase that is effective. The amount that can be dissolved in different foods depends on several different factors both intrinsic, like pH and fat content, and extrinsic, like storage temperature, gas/product volume ratio and

concentration of CO₂ in the gas phase (Devlieghere et al., 1998a). The solubility of CO₂ in the water phase, at moderate temperatures and pressures, closely follows Henry's law (Daniels et al., 1985):

$$[\text{CO}_2]_{\text{diss}} = K_{\text{H}} \times p\text{CO}_2$$

where K_{H} is Henry's law's constant and $p\text{CO}_2$ is the partial pressure of CO₂. Since K_{H} varies with temperature, more gas can be dissolved at lower temperatures. There also seems to be a synergistic effect between CO₂ and temperature, increasing the antimicrobial activity more than can be explained by just the increase in dissolved CO₂ (Devlieghere et al., 1998b).

In spite of all the advantages with MAP there is a valid concern that the modified atmosphere may inhibit spoilage organisms, while allowing or even promoting growth of pathogens. This might mean that food appears unspoiled and organoleptically acceptable, even long after the expiration date, while it is in fact toxic. This is especially a hazard in ready-to-eat foods which are not heated before consumption. The organisms that mainly cause concern in refrigerated foods are psychrotrophic foodborne pathogens such as *Listeria monocytogenes*, *Yersinia enterocolitica* and *Aeromonas hydrophila*, as well as non-proteolytic *C. botulinum*, although clearly a number of other microorganisms, especially *Salmonella* spp., *E. coli* O157:H7 and *Shigella* spp., can be potential health risks. Another type of food where MAP is considered hazardous is fresh fish. This is in part because of the high prevalence in fish of spores of *C. botulinum* type E. Since these non-proteolytic strains can produce toxin at temperatures as low as 3.3°C, and since maintenance of a storage temperature that is lower from time of packaging to consumption is not likely with current practices and abuses at the retail and consumer level, the US Food and Drug Administration (FDA) considers these products “a potentially life threatening acute health hazard because of the possibility of contamination with *C. botulinum* toxin”. They have ordered a detention without physical examination of refrigerated (not frozen), vacuum or modified atmosphere packaged raw fish and fishery products since “FDA considers refrigerated fresh fish products in vacuum packaging or modified atmosphere packaging to be adulterated under section 402(a)(4) of the Food, Drug and Cosmetic Act when the *C. botulinum* toxin hazard is not controlled” (IA #16-125 - 9/25/02). In Canada, regulation number B.21.025 of the Regulations Pursuant to the Food and Drugs Act states that “No person shall sell smoked marine and fresh water animals and their products or marine and fresh water animals and their products to which liquid smoke flavours has been added that is packed in a container sealed to exclude air unless it a) has been heat processed after sealing at a temperature and for a time sufficient to destroy all spores of the species *Clostridium botulinum*; or b) contains not less than 9 percent salt, as

determined by the official method; or c) is customarily cooked before eating; or d) is frozen and the label carries the following statement on the principal display panel in the same size type as used for the common name “Keep Frozen Prior to Use”.” Neither the EU in general nor Sweden has any rules limiting the use of CO₂ in food packaging. It is listed as substance generally permitted to be added to all foodstuffs in European Parliament and Council Directive No 95/2/EC on food additives other than colours and sweeteners and the Swedish law on food additives, LIVSFS 2004:30. The product needs to be marked “Packed in a protective atmosphere”, but the contents of the gas do not have to be specified.

Several investigations have shown that toxin production by *C. botulinum* can precede spoilage in such diverse foods as fish (Arritt et al., 2007), crumpets (Daifas et al., 1999a; Daifas et al., 1999b), rice (Kasai et al., 2005), sandwiches (Kautter et al., 1981), pork (Lambert et al., 1991), and sliced raw potatoes treated with NaHSO₃ (Solomon et al., 1998) when stored under modified atmosphere.

Effects of carbon dioxide on growth, toxin gene expression, and production of neurotoxin

While most studies have focused on the time to germination, growth, or toxin production under different modified atmospheres and at different temperatures (Kautter et al., 1981; Doyle, 1983; Foegeding and Busta, 1983; Lambert et al., 1991; Solomon et al., 1998; Daifas et al., 1999a; Daifas et al., 1999b; Gibson et al., 2000; Fernandez et al., 2001; Kasai et al., 2005; Arritt et al., 2007), we have concentrated on quantifying the neurotoxin expression and the amounts of toxin produced during different growth phases.

We found that the effect of carbon dioxide varies with the toxin type and possibly group. The effect on the expression of the different toxin types can be seen in Figure 12. While the maximum OD did not seem negatively affected, in non-proteolytic type B and E, growth rates decreased with increasing concentrations of CO₂ and lag phases increased (Papers II & III) (Figure 11).

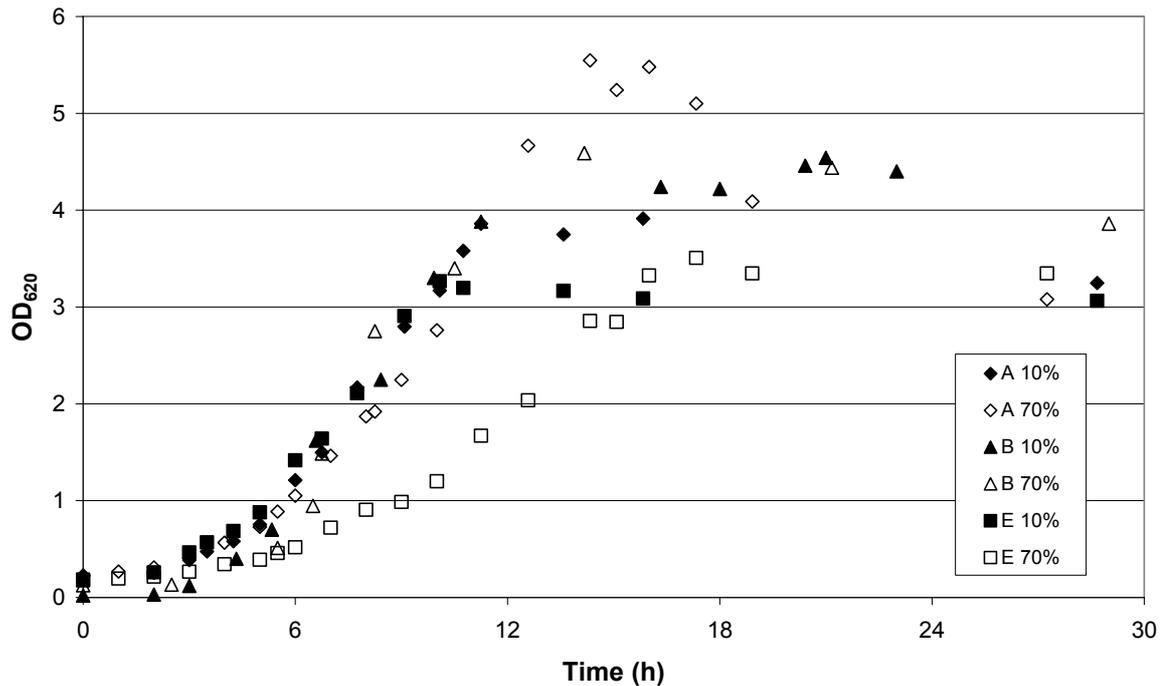


Figure 11 Effect of carbon dioxide on growth of *C. botulinum*. Diamonds – *C. botulinum* type A. Triangles – *C. botulinum* type B. Squares – *C. botulinum* type E. Filled symbols – 10% CO₂. Open symbols – 70% CO₂.

The neurotoxin gene expression, however, behaved in the opposite way, increasing with the level of CO₂, as did the amount of toxin produced. For *cntE* the expression in late exponential phase more than doubled when the concentration of CO₂ increased from 10% to 70%, while for *cntB* the effect was even more dramatic as it increased five-fold (Figure 12). The amount of toxin produced increased in a similar manner. While Sharkey et al. (Sharkey et al., 2004) claim that increased growth rate and cell mass production generally increases the levels of toxin encoding mRNA and people traditionally have believed that slowing growth will decrease toxin production, this phenomena is not completely unknown among clostridia. *Clostridium difficile* has been shown to produce more toxin when growth is slowed by stressful events (Moncrief et al., 1997). Since the expression of toxin in *C. difficile* is induced when it reaches stationary phase (Dupuy and Sonenshein, 1998), this may not be completely analogous. However, in both cases it has been suggested that one purpose of producing toxins may be to kill eukaryotic organisms, so the bacteria can utilise their nutrients and energy. Similar trends have been found in other genera as well. Expression of *emm*, the gene encoding M protein, and *scpA*, encoding the C5a endopeptidase Scp, which are major virulence factors in *Streptococcus pyogenes*, were stimulated by increased levels of CO₂ (Caparon et al., 1992; Stretton and Goodman, 1998). Carbon dioxide has also been found to stimulate toxin

production in *Staphylococcus aureus*, *Vibrio cholerae*, and *Bacillus anthracis* (Shimamura et al., 1985; Hoffmaster and Koehler, 1997; Stretton et al., 1998; Ross and Onderdonk, 2000; Drysdale et al., 2004; Drysdale et al., 2005).

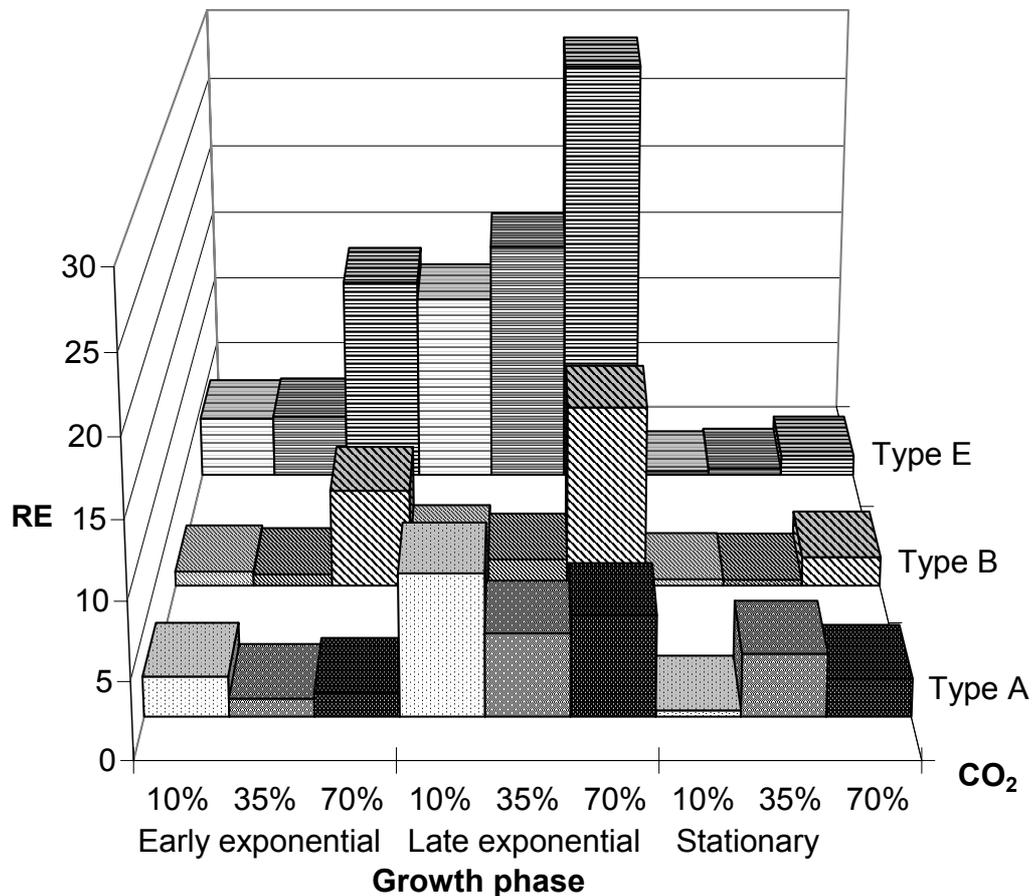


Figure 12 Effect of growth phase and carbon dioxide concentration on relative expression of neurotoxin in *C. botulinum*.

In the proteolytic type A, a completely different picture could be seen (Paper IV). Both the growth and the toxin expression seem unaffected by the amount of CO₂ in the atmosphere, even if the maximum OD seems to rise with increasing CO₂ levels. When looking at the overall expression of Hall A with microarrays, the trend is similar, but the expression of the neurotoxin cluster appears to continue rising, except for the *cntR* gene, which indicates that there may be something else affecting expression in the late stationary/death phase. While the increase in CO₂ does not seem to have a significant effect on the toxin expression, several other genes were induced at 35% and 70% CO₂. A list of these genes can be seen in Table 3. Several of these genes have been seen to be involved with stress response (Nagasawa et al., 1988; Kawasaki, 1993; Chen et al., 1998; Campobasso et al., 2000; Uo et al., 2002;

Goldsmith-Fischman et al., 2004; Glantzounis et al., 2005; Oliveira et al., 2005; Obiero et al., 2006; Schwarz and Mendel, 2006).

Table 3 Genes induced by growth at 35% and 70% CO₂.

| Locus | Protein |
|----------------|---|
| CBO2881 | Xanthine dehydrogenase, molybdenum-binding and iron-sulfur-binding subunits |
| CBO2882 | Peptidase |
| CBO2883 | Amidohydrolase |
| CBO2884 | Pyridine nucleotide-disulfide oxidoreductase |
| CBO2885 | Aspartate/ornithine carbamoyltransferase |
| CBO2886 | D-hydantoinase |
| CBO2887 | Xanthine permease |
| CBO2888 | Diaminopropionate ammonia-lyase |
| CBO1259 | Thioredoxin reductase |
| CBO1848 | Electron-transferring subunit of iron-only hydrogenase |
| CBO0450 | Hydroxyethylthiazole kinase |
| CBO2221 | Nitrogenase iron-molybdenum cofactor protein |
| CBO2222 | 4Fe-4S cluster containing ParA family ATPase protein |
| CBO2223 | 4Fe-4S cluster containing ParA family ATPase protein |

Concluding remarks

The research presented in this thesis has been focused on developing molecular methods for diagnosis of botulism and to increase the understanding of *Clostridium botulinum* and its neurotoxin formation. This knowledge can be used to improve food safety assessment, by seeing how different factors affect food preservation.

While methods to preserve food in a way that prevents botulism have been known for a long time, new consumer demands, new health issues, and new behavioural patterns create new problems. Today's consumers want food with little or no preservatives, salt, or sugar. This means that food producers try to cut down on salt and sodium nitrite, which has long been the combination of choice for preventing growth of *C. botulinum* in foods that could not be put through a botulinum cook (a 12D reduction in the amount of spores). At the same time the consumers want food with a long shelf life, that they can keep at home for a long time, without spoilage, and preferably without refrigeration. And lastly, they want food which looks and tastes fresh, with vitamins and other nutrient intact, which means it can not have undergone a harsh heat treatment. All this, taken together, has caused the food industry to try to find new methods of preserving food. While the new methods are tested for their ability to stop the growth of spoilage organisms, it is not certain that all types of pathogens are taken into account. As *C. botulinum* spores are present in a variety of places, including soil, fish, and the intestines of pigs and cattle (Dodds, 1993b; Dahlenborg et al., 2001; Dahlenborg et al., 2003), there is a high risk of contamination if the food is not handled correctly.

The safety of some of the new methods used for preservation, like MAP and vacuum packing, are dependent on the cold chain being kept. However, it is a well known fact that it is often broken, during transport to stores, by being left on the loading bay, by incorrect temperatures in the stores, and not least by the consumers themselves. Many consumers are under the impression that since something is vacuum packed it doesn't matter if it doesn't go straight in the refrigerator. Furthermore, most household refrigerators do not keep cold enough. Sous-vide (ready to eat, vacuum packed food) meat is e.g. supposed to be stored at a temperature below 3°C, a temperature seldom held in a household refrigerator.

In this work we found storing food under a high concentration of carbon dioxide can even increase the risk of botulism, since the expression and production of neurotoxin for non-proteolytic *C. botulinum* was enhanced, in spite of the delay in growth. Since these are the types that can grow at refrigeration temperatures, this can prove to be a great hazard. In the case of the proteolytic type A, neither growth nor toxin production was influenced. It would

be interesting, in the future, to see if this is a true difference between group I and II organisms, or something which varies between strains.

Finding new, quicker ways of identifying botulism can be effective in shortening the length and cost of hospital stay and the severity of illness. In addition, administration of antitoxin is most effective if done quickly. All types of botulism are probably underdiagnosed, partially because of problems in laboratory identification. In many cases, antibiotics are given before samples are taken, which prevents normal culture methods, while DNA-based methods can still be used. Because botulism is a relatively rare disease, delayed diagnosis is not uncommon. Because all types of botulism are relatively rare diseases and delayed diagnosis is not uncommon it is important that clinicians are aware of their existence.

Acknowledgements

I would like to thank everyone working at Medical Microbiology and Applied Microbiology for giving me a nice working environment and many good times.

I wish especially to thank:

My supervisors, **Elisabet Holst** and **Peter Rådström**, for giving me this opportunity, for their supervision, and for teaching me so many things.

Everybody at IFR, but especially **Mike Peck**, for letting me come, **Dave Mason**, for all the help in the lab, and **Andy Carter**, for taking such good care of a pregnant woman far away from home. I tried to return the favour when we got one here.

My old chemistry teacher, **Ingrid Ohlén**, who got me interested in chemistry in general and gene technology in particular.

Everybody in the old PCR-group, thank you for the fun on +4, in Norway, and other places. Thank you for still helping me even after you finished.

My new roommates, for help in finding tricks in how the computer works and creating a nice environment.

My undergraduate students, **Sophie**, **Camilla**, **Gun**, and **Anna** for your contributions to this work.

Ed, **Ted** and **Boaz** for nice lunches and entertaining existential discussions.

Christer, for helping me with pictures and other technical problems.

Birgit, without whom the department doesn't run.

The girl gang, for putting up with me never having any time and making sure I do something other than work. Thank you for all the fun, the help, and the support.

All my friends and family.

My mother, **Kirsten**, for always being there for me, with love, help, and support.

My husband **Jens**, for taking care of everything at home, for putting up with me coming home late all the time, and for all your love and support. I couldn't have done this without you.

And of course, my son **Viggo**, for being the light of my life.

THANK YOU!

Ingrid

This work was supported by the Swedish Governmental Agency for Innovation Systems (VINNOVA), the Swedish Research Council for Environment, Agricultural Sciences, and Special Planning (Formas) as well as by grants from the Competitive Strategic Grant of the BBSRC, and by a Marie Curie training site fellowship. My visits to conferences to present my work has been supported by grants from LivsmedelsFöreningen, Medicinska fakultetens forskarutbildningskommitté, and Kungliga Fysiografiska Sällskapet i Lund.

References

- Abgueuen, P., V. Delbos, J. M. Chennebault, S. Fanello, O. Brenet, P. Alquier, J. C. Granry and E. Pichard (2003). "Nine cases of foodborne botulism type B in France and literature review." *Eur J Clin Microbiol Infect Dis* **22**(12): 749-52.
- Abu Al-Soud, W. and P. Rådström (1998). "Capacity of nine thermostable DNA polymerases To mediate DNA amplification in the presence of PCR-inhibiting samples." *Appl Environ Microbiol* **64**(10): 3748-53.
- Abu Al-Soud, W. and P. Rådström (2000). "Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat." *J Clin Microbiol* **38**(12): 4463-70.
- Ahmed, S. A., M. P. Byrne, M. Jensen, H. B. Hines, E. Brueggemann and L. A. Smith (2001). "Enzymatic autocatalysis of botulinum A neurotoxin light chain." *J Protein Chem* **20**(3): 221-31.
- Ahnert-Hilger, G. and H. Bigalke (1995). "Molecular aspects of tetanus and botulinum neurotoxin poisoning." *Prog Neurobiol* **46**(1): 83-96.
- Akbulut, D., J. Dennis, M. Gent, K. Grant, V. Hope, C. Ohai, J. McLauchlin, V. Mithani, O. Mпамуго, F. Ncube and L. De Souza-Thomas (2005). "Wound botulism in injectors of drugs: upsurge in cases in England during 2004." *Euro Surveill* **10**(9).
- Akbulut, D., K. A. Grant and J. McLauchlin (2004). "Development and application of Real-Time PCR assays to detect fragments of the *Clostridium botulinum* types A, B, and E neurotoxin genes for investigation of human foodborne and infant botulism." *Foodborne Pathog Dis* **1**(4): 247-57.
- Alpers, K., U. van Treeck and C. Frank (2005). "Outbreak of wound botulism in injecting drug users in Germany, October-December 2005." *Euro Surveill* **10**(12): E051215 4.

- Alsallami, A. A. and R. Kotlowski (2001). "Selection of primers for specific detection of *Clostridium botulinum* types B and E neurotoxin genes using PCR method." *Int J Food Microbiol* **69**(3): 247-53.
- Angulo, F. J., J. Getz, J. P. Taylor, K. A. Hendricks, C. L. Hatheway, S. S. Barth, H. M. Solomon, A. E. Larson, E. A. Johnson, L. N. Nickey and A. A. Ries (1998). "A large outbreak of botulism: the hazardous baked potato." *J Infect Dis* **178**(1): 172-7.
- Anonymous (1991). "Botulism." *Epid-aktuell* **14**(6): 9.
- Aranda, E., M. M. Rodriguez, M. A. Asensio and J. J. Cordoba (1997). "Detection of *Clostridium botulinum* types A, B, E and F in foods by PCR and DNA probe." *Lett Appl Microbiol* **25**(3): 186-90.
- Arnon, S. S., R. Schechter, T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, J. Hauer, M. Layton, S. Lillibridge, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, D. L. Swerdlow and K. Tonat (2001). "Botulinum toxin as a biological weapon: medical and public health management." *Jama* **285**(8): 1059-70.
- Arnon, S. S., R. Schechter, S. E. Maslanka, N. P. Jewell and C. L. Hatheway (2006). "Human botulism immune globulin for the treatment of infant botulism." *N Engl J Med* **354**(5): 462-71.
- Arritt, F. M., J. D. Eifert, M. L. Jahncke, M. D. Pierson and R. C. Williams (2007). "Effects of modified atmosphere packaging on toxin production by *Clostridium botulinum* in raw aquacultured summer flounder fillets (*Paralichthys dentatus*)." *J Food Prot* **70**(5): 1159-64.
- Aureli, P., M. Di Cunto, A. Maffei, G. De Chiara, G. Franciosa, L. Accorinti, A. M. Gambardella and D. Greco (2000). "An outbreak in Italy of botulism associated with a dessert made with mascarpone cream cheese." *Eur J Epidemiol* **16**(10): 913-8.

- Bade, S., A. Rummel, C. Reisinger, T. Karnath, G. Ahnert-Hilger, H. Bigalke and T. Binz (2004). "Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates." *J Neurochem* **91**(6): 1461-72.
- Baltimore, D. (1970). "RNA-dependent DNA polymerase in virions of RNA tumour viruses." *Nature* **226**(5252): 1209-11.
- Becker-Andre, M. and K. Hahlbrock (1989). "Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY)." *Nucleic Acids Res* **17**(22): 9437-46.
- Bell, C. and A. Kyriakides (2000). *Clostridium botulinum: A Practical Approach to the Organism and its Control in Foods*. London, Blackwell Science Ltd.
- Binz, T., H. Kurazono, M. R. Popoff, M. W. Eklund, G. Sakaguchi, S. Kozaki, K. Krieglstein, A. Henschen, D. M. Gill and H. Niemann (1990a). "Nucleotide sequence of the gene encoding *Clostridium botulinum* neurotoxin type D." *Nucleic Acids Res* **18**(18): 5556.
- Binz, T., H. Kurazono, M. Wille, J. Frevert, K. Wernars and H. Niemann (1990b). "The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins." *J Biol Chem* **265**(16): 9153-8.
- Bohnel, H., S. Behrens, P. Loch, K. Lube and F. Gessler (2001). "Is there a link between infant botulism and sudden infant death? Bacteriological results obtained in central Germany." *Eur J Pediatr* **160**(10): 623-8.
- Bradshaw, M., S. S. Dineen, N. D. Maks and E. A. Johnson (2004). "Regulation of neurotoxin complex expression in *Clostridium botulinum* strains 62A, Hall A-hyper, and NCTC 2916." *Anaerobe* **10**(6): 321-33.
- Brett, M. (1999). "Botulism in the United Kingdom." *Euro Surveill* **4**(1): 9-11.

- Brett, M. M., G. Hallas and O. Mpamugo (2004). "Wound botulism in the UK and Ireland." *J Med Microbiol* **53**(Pt 6): 555-61.
- Brett, M. M., J. McLauchlin, A. Harris, S. O'Brien, N. Black, R. J. Forsyth, D. Roberts and F. J. Bolton (2005). "A case of infant botulism with a possible link to infant formula milk powder: evidence for the presence of more than one strain of *Clostridium botulinum* in clinical specimens and food." *J Med Microbiol* **54**(Pt 8): 769-76.
- Brook, I. (2007). "Infant botulism." *J Perinatol* **27**(3): 175-80.
- Bustin, S. A. (2000). "Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays." *J Mol Endocrinol* **25**(2): 169-93.
- Bustin, S. A. (2002). "Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems." *J Mol Endocrinol* **29**(1): 23-39.
- Bustin, S. A., V. Benes, T. Nolan and M. W. Pfaffl (2005). "Quantitative real-time RT-PCR-- a perspective." *J Mol Endocrinol* **34**(3): 597-601.
- Cai, S., B. R. Singh and S. Sharma (2007). "Botulism Diagnostics: From Clinical Symptoms to in vitro Assays." *Crit Rev Microbiol* **33**(2): 109-25.
- Campbell, K., M. D. Collins and A. K. East (1993). "Nucleotide sequence of the gene coding for *Clostridium botulinum* (*Clostridium argentinense*) type G neurotoxin: genealogical comparison with other clostridial neurotoxins." *Biochim Biophys Acta* **1216**(3): 487-91.
- Campobasso, N., Mathews, II, T. P. Begley and S. E. Ealick (2000). "Crystal structure of 4-methyl-5-beta-hydroxyethylthiazole kinase from *Bacillus subtilis* at 1.5 Å resolution." *Biochemistry* **39**(27): 7868-77.
- Caparon, M. G., R. T. Geist, J. Perez-Casal and J. R. Scott (1992). "Environmental regulation of virulence in group A streptococci: transcription of the gene encoding M protein is stimulated by carbon dioxide." *J Bacteriol* **174**(17): 5693-701.

- CDC (1992). "Outbreak of type E botulism associated with an uneviscerated, salt-cured fish product--New Jersey, 1992." MMWR Morb Mortal Wkly Rep **41**(29): 521-2.
- CDC (1995a). "Foodborne botulism--Oklahoma, 1994." MMWR Morb Mortal Wkly Rep **44**(11): 200-2.
- CDC (1995b). "Type B botulism associated with roasted eggplant in oil--Italy, 1993." MMWR Morb Mortal Wkly Rep **44**(2): 33-6.
- CDC (1998). Botulism in the United States, 1899-1996. Handbook for Epidemiologists, Clinicians, and Laboratory Workers. Atlanta, GA, Centers for Disease Control and Prevention.
- CDC (1999). "Foodborne botulism associated with home-canned bamboo shoots--Thailand, 1998." MMWR Morb Mortal Wkly Rep **48**(21): 437-9.
- CDC (2000). "Foodborne botulism from eating home-pickled eggs--Illinois, 1997." MMWR Morb Mortal Wkly Rep **49**(34): 778-80.
- CDC (2001). "Botulism outbreak associated with eating fermented food--Alaska, 2001." MMWR Morb Mortal Wkly Rep **50**(32): 680-2.
- CDC (2003). "Outbreak of botulism type E associated with eating a beached whale--Western Alaska, July 2002." MMWR Morb Mortal Wkly Rep **52**(2): 24-6.
- CDC (2006a). "Botulism associated with commercial carrot juice--Georgia and Florida, September 2006." MMWR Morb Mortal Wkly Rep **55**(40): 1098-9.
- CDC (2006b). "Botulism from home-canned bamboo shoots--Nan Province, Thailand, March 2006." MMWR Morb Mortal Wkly Rep **55**(14): 389-92.
- CDC (2007a). "Botulism associated with commercially canned chili sauce--Texas and Indiana, July 2007." MMWR Morb Mortal Wkly Rep **56**(30): 767-9.

- CDC (2007b). "Foodborne botulism from home-prepared fermented tofu--California, 2006." *MMWR Morb Mortal Wkly Rep* **56**(5): 96-7.
- Cengiz, M., M. Yilmaz, L. Dosemeci and A. Ramazanoglu (2006). "A botulism outbreak from roasted canned mushrooms." *Hum Exp Toxicol* **25**(5): 273-8.
- Chaffer, M., M. Baum, K. Grinberg, T. Molad and D. Elad (2006). "Application of PCR for detection of *Clostridium botulinum* type D in bovine samples." *J Vet Med B Infect Dis Vet Public Health* **53**(1): 45-7.
- Chang, G. Y. and G. Ganguly (2003). "Early antitoxin treatment in wound botulism results in better outcome." *Eur Neurol* **49**(3): 151-3.
- Chaudhry, R., B. Dhawan, D. Kumar, R. Bhatia, J. C. Gandhi, R. K. Patel and B. C. Purohit (1998). "Outbreak of suspected *Clostridium butyricum* botulism in India." *Emerg Infect Dis* **4**(3): 506-7.
- Chehab, F. F. and Y. W. Kan (1989). "Detection of specific DNA sequences by fluorescence amplification: a color complementation assay." *Proc Natl Acad Sci U S A* **86**(23): 9178-82.
- Chelly, J., J. C. Kaplan, P. Maire, S. Gautron and A. Kahn (1988). "Transcription of the dystrophin gene in human muscle and non-muscle tissue." *Nature* **333**(6176): 858-60.
- Chen, K. H., F. M. Yakes, D. K. Srivastava, R. K. Singhal, R. W. Sobol, J. K. Horton, B. Van Houten and S. H. Wilson (1998). "Up-regulation of base excision repair correlates with enhanced protection against a DNA damaging agent in mouse cell lines." *Nucleic Acids Res* **26**(8): 2001-7.
- Chen, S., J. J. Kim and J. T. Barbieri (2007). "Mechanism of substrate recognition by botulinum neurotoxin serotype A." *J Biol Chem* **282**(13): 9621-7.
- Cherington, M. (1998). "Clinical spectrum of botulism." *Muscle Nerve* **21**(6): 701-10.

- Chertow, D. S., E. T. Tan, S. E. Maslanka, J. Schulte, E. A. Bresnitz, R. S. Weisman, J. Bernstein, S. M. Marcus, S. Kumar, J. Malecki, J. Sobel and C. R. Braden (2006). "Botulism in 4 adults following cosmetic injections with an unlicensed, highly concentrated botulinum preparation." *Jama* **296**(20): 2476-9.
- Christensen, D. R., L. J. Hartman, B. M. Loveless, M. S. Frye, M. A. Shipley, D. L. Bridge, M. J. Richards, R. S. Kaplan, J. Garrison, C. D. Baldwin, D. A. Kulesh and D. A. Norwood (2006). "Detection of biological threat agents by real-time PCR: comparison of assay performance on the R.A.P.I.D., the LightCycler, and the Smart Cycler platforms." *Clin Chem* **52**(1): 141-5.
- Clegg, R. M. (1992). "Fluorescence resonance energy transfer and nucleic acids." *Methods Enzymol* **211**: 353-88.
- Cockerill, F. R., 3rd (2003). "Application of rapid-cycle real-time polymerase chain reaction for diagnostic testing in the clinical microbiology laboratory." *Arch Pathol Lab Med* **127**(9): 1112-20.
- Couesnon, A., S. Raffestin and M. R. Popoff (2006). "Expression of botulinum neurotoxins A and E, and associated non-toxin genes, during the transition phase and stability at high temperature: analysis by quantitative reverse transcription-PCR." *Microbiology* **152**(Pt 3): 759-70.
- Cox, N. and R. Hinkle (2002). "Infant botulism." *Am Fam Physician* **65**(7): 1388-92.
- Culliton, B. J. (1971). "Reverse transcription: one year later." *Science* **172**(986): 926-8.
- Dahlenborg, M., E. Borch and P. Rådström (2003). "Prevalence of *Clostridium botulinum* types B, E and F in faecal samples from Swedish cattle." *Int J Food Microbiol* **82**(2): 105-10.
- Dahlenborg, M., E. Borch and P. Rådström (2001). "Development of a Combined Selection and Enrichment PCR Procedure for *Clostridium botulinum* Types B, E, and F and Its

- Use To Determine Prevalence in Fecal Samples from Slaughtered Pigs." *Appl Environ Microbiol* **67**(10): 4781-8.
- Daifas, D. P., J. P. Smith, B. Blanchfield and J. W. Austin (1999a). "Effect of pH and CO₂ on growth and toxin production by *Clostridium botulinum* in English-style crumpets packaged under modified atmospheres." *J Food Prot* **62**(10): 1157-61.
- Daifas, D. P., J. P. Smith, B. Blanchfield and J. W. Austin (1999b). "Growth and toxin production by *Clostridium botulinum* in English-style crumpets packaged under modified atmospheres." *J Food Prot* **62**(4): 349-55.
- Dancer, S. J., D. McNair, P. Finn and A. B. Kolsto (2002). "*Bacillus cereus* cellulitis from contaminated heroin." *J Med Microbiol* **51**(3): 278-81.
- Daniels, J. A., R. Krishnamurthi and S. S. Rizvi (1985). "A review of effects of carbon dioxide on microbial growth and food quality." *Journal of Food Protection* **48**(6): 532-537.
- Dawar, M., L. Moody, J. D. Martin, C. Fung, J. Isaac-Renton and D. M. Patrick (2002). "Two outbreaks of botulism associated with fermented salmon roe--British Columbia, August 2001." *Can Commun Dis Rep* **28**(6): 45-9.
- Delidow, B. C., J. J. Peluso and B. A. White (1989). "Quantitative measurement of mRNAs by polymerase chain reaction." *Gene Anal Tech* **6**(6): 120-4.
- DeRisi, J. L., V. R. Iyer and P. O. Brown (1997). "Exploring the metabolic and genetic control of gene expression on a genomic scale." *Science* **278**(5338): 680-6.
- Devlieghere, F., J. Debevere and J. Van Impe (1998a). "Concentration of carbon dioxide in the water-phase as a parameter to model the effect of a modified atmosphere on microorganisms." *Int J Food Microbiol* **43**(1-2): 105-13.

- Devlieghere, F., J. Debevere and J. Van Impe (1998b). "Effect of dissolved carbon dioxide and temperature on the growth of *Lactobacillus sake* in modified atmospheres." *Int J Food Microbiol* **41**(3): 231-8.
- Dixon, N. M. and D. B. Kell (1989). "The inhibition by CO₂ of the growth and metabolism of micro-organisms." *J Appl Bacteriol* **67**(2): 109-36.
- Dodds, K. L. (1993a). *Clostridium botulinum* in foods. *Clostridium botulinum. Ecology and control in foods.* A. H. W. Hauschild and K. L. Dodds. New York, Marcel Dekker, Inc. **54**: 53-68.
- Dodds, K. L. (1993b). *Clostridium botulinum* in the environment. *Clostridium botulinum. Ecology and control in foods.* A. H. W. Hauschild and K. L. Dodds. New York, Marcel Dekker, Inc. **54**: 21-51.
- Dodds, K. L. (1993c). Worldwide incidence and ecology of infant botulism. *Clostridium botulinum. Ecology and control in foods.* A. H. W. Hauschild and K. L. Dodds. New York, Marcel Dekker, Inc. **54**: 105-117.
- Doellgast, G. J., M. X. Triscott, G. A. Beard, J. D. Bottoms, T. Cheng, B. H. Roh, M. G. Roman, P. A. Hall and J. E. Brown (1993). "Sensitive enzyme-linked immunosorbent assay for detection of *Clostridium botulinum* neurotoxins A, B, and E using signal amplification via enzyme-linked coagulation assay." *J Clin Microbiol* **31**(9): 2402-9.
- Dong, M., W. H. Tepp, E. A. Johnson and E. R. Chapman (2004). "Using fluorescent sensors to detect botulinum neurotoxin activity in vitro and in living cells." *Proc Natl Acad Sci U S A* **101**(41): 14701-6.
- Doyle, M. P. (1983). "Effect of carbon dioxide on toxin production by *Clostridium botulinum*." *European journal of applied microbiology and biotechnology* **17**(1): 53-56.
- Drysdale, M., A. Bourgogne, S. G. Hilsenbeck and T. M. Koehler (2004). "*atxA* controls *Bacillus anthracis* capsule synthesis via *acpA* and a newly discovered regulator, *acpB*." *J Bacteriol* **186**(2): 307-15.

- Drysdale, M., A. Bourgoigne and T. M. Koehler (2005). "Transcriptional analysis of the *Bacillus anthracis* capsule regulators." *J Bacteriol* **187**(15): 5108-14.
- Duggan, D. J., M. Bittner, Y. Chen, P. Meltzer and J. M. Trent (1999). "Expression profiling using cDNA microarrays." *Nat Genet* **21**(1 Suppl): 10-4.
- Dupuy, B. and A. L. Sonenshein (1998). "Regulated transcription of *Clostridium difficile* toxin genes." *Mol Microbiol* **27**(1): 107-20.
- East, A. K., M. Bhandari, J. M. Stacey, K. D. Campbell and M. D. Collins (1996). "Organization and phylogenetic interrelationships of genes encoding components of the botulinum toxin complex in proteolytic *Clostridium botulinum* types A, B, and F: evidence of chimeric sequences in the gene encoding the nontoxic nonhemagglutinin component." *Int J Syst Bacteriol* **46**(4): 1105-12.
- East, A. K., P. T. Richardson, D. Allaway, M. D. Collins, T. A. Roberts and D. E. Thompson (1992). "Sequence of the gene encoding type F neurotoxin of *Clostridium botulinum*." *FEMS Microbiol Lett* **75**(2-3): 225-30.
- Eklund, M. W. and F. T. Poysky (1974). "Interconversion of type C and D strains of *Clostridium botulinum* by specific bacteriophages." *Appl Microbiol* **27**(1): 251-8.
- Eklund, M. W., F. T. Poysky, L. M. Mseitif and M. S. Strom (1988). "Evidence for plasmid-mediated toxin and bacteriocin production in *Clostridium botulinum* type G." *Appl Environ Microbiol* **54**(6): 1405-8.
- Eleaume, H. and S. Jabbouri (2004). "Comparison of two standardisation methods in real-time quantitative RT-PCR to follow *Staphylococcus aureus* genes expression during in vitro growth." *J Microbiol Methods* **59**(3): 363-70.
- Erbguth, F. J. and M. Naumann (1999). "Historical aspects of botulinum toxin: Justinus Kerner (1786-1862) and the "sausage poison"." *Neurology* **53**(8): 1850-3.

- Fach, P., M. Gibert, R. Griffais, J. P. Guillou and M. R. Popoff (1995). "PCR and gene probe identification of botulinum neurotoxin A-, B-, E-, F-, and G-producing *Clostridium spp.* and evaluation in food samples." *Appl Environ Microbiol* **61**(1): 389-92.
- Farber, J. M. (1991). "Microbiological aspects of modified-atmosphere packaging technology - A review." *Journal of Food Protection* **54**(1): 58-70.
- Fenicia, L., F. Anniballi, D. De Medici, E. Delibato and P. Aureli (2007). "SYBR green real-time PCR method to detect *Clostridium botulinum* type A." *Appl Environ Microbiol* **73**(9): 2891-6.
- Fernandez, P. S., J. Baranyi and M. W. Peck (2001). "A predictive model of growth from spores of non-proteolytic *Clostridium botulinum* in the presence of different CO₂ concentrations as influenced by chill temperature, pH and NaCl." *Food Microbiology* **18**: 453-461.
- Ferreira, J. L., S. Maslanka, E. Johnson and M. Goodnough (2003). "Detection of botulinum neurotoxins A, B, E, and F by amplified enzyme-linked immunosorbent assay: collaborative study." *J AOAC Int* **86**(2): 314-31.
- Foegeding, P. M. and F. F. Busta (1983). "Effect of carbon dioxide, nitrogen and hydrogen gases on germination of *Clostridium botulinum* spores." *Journal of Food Protection* **46**(11): 987-989.
- Franciosa, G., L. Fenicia, M. Pourshaban and P. Aureli (1997). "Recovery of a strain of *Clostridium botulinum* producing both neurotoxin A and neurotoxin B from canned macrobiotic food." *Appl Environ Microbiol* **63**(3): 1148-50.
- Franciosa, G., J. L. Ferreira and C. L. Hatheway (1994). "Detection of type A, B, and E botulinum neurotoxin genes in *Clostridium botulinum* and other *Clostridium* species by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms." *J Clin Microbiol* **32**(8): 1911-7.

- Frean, J., L. Arntzen, J. van den Heever and O. Perovic (2004). "Fatal type A botulism in South Africa, 2002." *Trans R Soc Trop Med Hyg* **98**(5): 290-5.
- Gessler, F., S. Pagel-Wieder, M. A. Avondet and H. Bohnel (2007). "Evaluation of lateral flow assays for the detection of botulinum neurotoxin type A and their application in laboratory diagnosis of botulism." *Diagn Microbiol Infect Dis* **57**(3): 243-9.
- Gibson, A. M., R. C. Ellis-Brownlee, M. E. Cahill, E. A. Szabo, G. C. Fletcher and P. J. Bremer (2000). "The effect of 100% CO₂ on the growth of nonproteolytic *Clostridium botulinum* at chill temperatures." *Int J Food Microbiol* **54**(1-2): 39-48.
- Giulietti, A., L. Overbergh, D. Valckx, B. Decallonne, R. Bouillon and C. Mathieu (2001). "An Overview of Real-Time Quantitative PCR: Applications to Quantify Cytokine Gene Expression." *Methods* **25**(4): 386-401.
- Glantzounis, G. K., E. C. Tsimoyiannis, A. M. Kappas and D. A. Galaris (2005). "Uric acid and oxidative stress." *Curr Pharm Des* **11**(32): 4145-51.
- Goerke, C., M. G. Bayer and C. Wolz (2001). "Quantification of bacterial transcripts during infection using competitive reverse transcription-PCR (RT-PCR) and LightCycler RT-PCR." *Clin Diagn Lab Immunol* **8**(2): 279-82.
- Goldsmith-Fischman, S., A. Kuzin, W. C. Edstrom, J. Benach, R. Shastry, R. Xiao, T. B. Acton, B. Honig, G. T. Montelione and J. F. Hunt (2004). "The SufE sulfur-acceptor protein contains a conserved core structure that mediates interdomain interactions in a variety of redox protein complexes." *J Mol Biol* **344**(2): 549-65.
- Graham, A. F., D. R. Mason, F. J. Maxwell and M. W. Peck (1997). "Effect of pH and NaCl on growth from spores of non-proteolytic *Clostridium botulinum* at chill temperature." *Lett Appl Microbiol* **24**(2): 95-100.
- Hallis, B., B. A. James and C. C. Shone (1996). "Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities." *J Clin Microbiol* **34**(8): 1934-8.

- Harvey, S. M., J. Sturgeon and D. E. Dassey (2002). "Botulism Due to *Clostridium baratii* Type F Toxin." J Clin Microbiol **40**(6): 2260-2.
- Hatheway, C. L. (1993). *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin. *Clostridium botulinum: Ecology and control in foods*. A. H. W. Hauschild and K. L. Dodds. New York, Marcel Dekker, Inc.: 3-20.
- Hielm, S., E. Hyytia, J. Ridell and H. Korkeala (1996). "Detection of *Clostridium botulinum* in fish and environmental samples using polymerase chain reaction." Int J Food Microbiol **31**(1-3): 357-65.
- Higuchi, R., G. Dollinger, P. S. Walsh and R. Griffith (1992). "Simultaneous amplification and detection of specific DNA sequences." Biotechnology (N Y) **10**(4): 413-7.
- Higuchi, R., C. Fockler, G. Dollinger and R. Watson (1993). "Kinetic PCR analysis: real-time monitoring of DNA amplification reactions." Biotechnology (N Y) **11**(9): 1026-30.
- Hill, K. K., T. J. Smith, C. H. Helma, L. O. Ticknor, B. T. Foley, R. T. Svensson, J. L. Brown, E. A. Johnson, L. A. Smith, R. T. Okinaka, P. J. Jackson and J. D. Marks (2007). "Genetic diversity among Botulinum Neurotoxin-producing clostridial strains." J Bacteriol **189**(3): 818-32.
- Hoffmaster, A. R. and T. M. Koehler (1997). "The anthrax toxin activator gene *atxA* is associated with CO₂-enhanced non-toxin gene expression in *Bacillus anthracis*." Infect Immun **65**(8): 3091-9.
- Holdeman, L. V., E. P. Cato and W. E. C. Moore (1977). VPI Anaerobe Laboratory Manual. Virginia Polytechnique Institute. Blacksburg, Va, U.S.A., State University.
- Holland, P. M., R. D. Abramson, R. Watson and D. H. Gelfand (1991). "Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of *Thermus aquaticus* DNA polymerase." Proc Natl Acad Sci U S A **88**(16): 7276-80.

- Hoorfar, J., B. Malorny, A. Abdulmawjood, N. Cook, M. Wagner and P. Fach (2004). "Practical considerations in design of internal amplification controls for diagnostic PCR assays." *J Clin Microbiol* **42**(5): 1863-8.
- Humeau, Y., F. Doussau, N. J. Grant and B. Poulain (2000). "How botulinum and tetanus neurotoxins block neurotransmitter release." *Biochimie* **82**(5): 427-46.
- Jensen, T., D. Jacobsen, E. von der Lippe, M. S. Heier and B. Selseth (1998). "[Clinical wound botulism in injecting drug addicts]." *Tidsskr Nor Laegeforen* **118**(28): 4363-5.
- Jensenius, M., R. Z. Lovstad, G. Dhaenens and L. M. Rorvik (2000). "A heroin user with a wobbly head." *Lancet* **356**(9236): 1160.
- Johnson, E. A. and M. Bradshaw (2001). "*Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective." *Toxicon* **39**(11): 1703-22.
- Jung, R., K. Soondrum and M. Neumaier (2000). "Quantitative PCR." *Clin Chem Lab Med* **38**(9): 833-6.
- Kacian, D. L., K. F. Watson, A. Burny and S. Spiegelman (1971). "Purification of the DNA polymerase of avian myeloblastosis virus." *Biochim Biophys Acta* **246**(3): 365-83.
- Kalb, S. R., H. Moura, A. E. Boyer, L. G. McWilliams, J. L. Pirkle and J. R. Barr (2006). "The use of Endopep-MS for the detection of botulinum toxins A, B, E, and F in serum and stool samples." *Anal Biochem* **351**(1): 84-92.
- Kalluri, P., C. Crowe, M. Reller, L. Gaul, J. Hayslett, S. Barth, S. Eliasberg, J. Ferreira, K. Holt, S. Bengston, K. Hendricks and J. Sobel (2003). "An outbreak of foodborne botulism associated with food sold at a salvage store in Texas." *Clin Infect Dis* **37**(11): 1490-5.
- Kasai, Y., B. Kimura, S. Kawasaki, T. Fukaya, K. Sakuma and T. Fujii (2005). "Growth and toxin production by *Clostridium botulinum* in steamed rice aseptically packed under modified atmosphere." *J Food Prot* **68**(5): 1005-11.

- Kautter, D. A., R. K. Lynt, T. Lilly and H. M. Solomon (1981). "Evaluation of botulism hazard from nitrogen-packed sandwiches." *Journal of Food Protection* **44**(1): 59-61.
- Kawasaki, Y. (1993). "Copurification of hydroxyethylthiazole kinase and thiamine-phosphate pyrophosphorylase of *Saccharomyces cerevisiae*: characterization of hydroxyethylthiazole kinase as a bifunctional enzyme in the thiamine biosynthetic pathway." *J Bacteriol* **175**(16): 5153-8.
- Kimura, B., S. Kawasaki, H. Nakano and T. Fujii (2001). "Rapid, quantitative PCR monitoring of growth of *Clostridium botulinum* type E in modified-atmosphere-packaged fish." *Appl Environ Microbiol* **67**(1): 206-16.
- Kimura, K., N. Fujii, K. Tsuzuki, T. Murakami, T. Indoh, N. Yokosawa, K. Takeshi, B. Syuto and K. Oguma (1990). "The complete nucleotide sequence of the gene coding for botulinum type C1 toxin in the C-ST phage genome." *Biochem Biophys Res Commun* **171**(3): 1304-11.
- Klein, D., P. Janda, R. Steinborn, M. Muller, B. Salmons and W. H. Gunzburg (1999). "Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: influence of mismatches on quantification." *Electrophoresis* **20**(2): 291-9.
- Koriazova, L. K. and M. Montal (2003). "Translocation of botulinum neurotoxin light chain protease through the heavy chain channel." *Nat Struct Biol* **10**(1): 13-8.
- Korkeala, H., G. Stengel, E. Hyytia, B. Vogelsang, A. Bohl, H. Wihlman, P. Pakkala and S. Hielm (1998). "Type E botulism associated with vacuum-packaged hot-smoked whitefish." *Int J Food Microbiol* **43**(1-2): 1-5.
- Kouguchi, H., T. Suzuki, K. Hasegawa, S. Mutoh, T. Watanabe, K. Niwa, T. Yoneyama, Y. Katoh and T. Ohyama (2006). "Quantitative detection of gene expression and toxin complex produced by *Clostridium botulinum* serotype D strain 4947." *J Microbiol Methods* **67**(3): 416-23.

- Kubota, T., N. Yonekura, Y. Hariya, E. Isogai, H. Isogai, K. Amano and N. Fujii (1998). "Gene arrangement in the upstream region of *Clostridium botulinum* type E and *Clostridium butyricum* BL6340 progenitor toxin genes is different from that of other types." FEMS Microbiol Lett **158**(2): 215-21.
- Kuusi, M., V. Hasseltvedt and P. Aavitsland (1999). "Botulism in Norway." Euro Surveill **4**(1): 11-12.
- Lacy, D. B., W. Tepp, A. C. Cohen, B. R. DasGupta and R. C. Stevens (1998). "Crystal structure of botulinum neurotoxin type A and implications for toxicity." Nat Struct Biol **5**(10): 898-902.
- Lambert, A. D., J. P. Smith and K. L. Dodds (1991). "Effect of headspace CO₂ concentration on toxin production by *Clostridium botulinum* in MAP, irradiated fresh pork." J Food Protection **54**(8): 588-592.
- Lantz, P. G., W. Abu al-Soud, R. Knutsson, B. Hahn-Hägerdal and P. Rådström (2000). "Biotechnical use of polymerase chain reaction for microbiological analysis of biological samples." Biotechnol Annu Rev **5**: 87-130.
- Lindström, M., S. Hielm, M. Nevas, S. Tuisku and H. Korkeala (2004). "Proteolytic *Clostridium botulinum* type B in the gastric content of a patient with type E botulism due to whitefish eggs." Foodborne Pathog Dis **1**(1): 53-7.
- Lindström, M., R. Keto, A. Markkula, M. Nevas, S. Hielm and H. Korkeala (2001). "Multiplex PCR Assay for Detection and Identification of *Clostridium botulinum* Types A, B, E, and F in Food and Fecal Material." Appl Environ Microbiol **67**(12): 5694-9.
- Lindström, M. and H. Korkeala (2006a). "Laboratory diagnostics of botulism." Clin Microbiol Rev **19**(2): 298-314.

- Lindström, M., M. Vuorela, K. Hinderink, H. Korkeala, E. Dahlsten, M. Raahenmaa and M. Kuusi (2006b). "Botulism associated with vacuum-packed smoked whitefish in Finland, June-July 2006." *Euro Surveill* **11**(7): E060720 3.
- Lindström, M. K., H. M. Jankola, S. Hielm, E. K. Hyytiä and H. J. Korkeala (1999). "Identification of *Clostridium botulinum* with API 20 A, Rapid ID 32 A and RapID ANA II." *FEMS Immunol Med Microbiol* **24**(3): 267-74.
- Livak, K. J., S. J. Flood, J. Marmaro, W. Giusti and K. Deetz (1995). "Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization." *PCR Methods Appl* **4**(6): 357-62.
- Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." *Methods* **25**(4): 402-8.
- Lohse, N., P. G. Kraghede and K. Molbak (2003). "[Botulism an a 38-year-old man after ingestion of garlic in chilli oil]." *Ugeskr Laeger* **165**(30): 2962-3.
- Lucchini, S., A. Thompson and J. C. Hinton (2001). "Microarrays for microbiologists." *Microbiology* **147**(Pt 6): 1403-14.
- Löfström, C., R. Knutsson, C. E. Axelsson and P. Rådström (2004). "Rapid and specific detection of *Salmonella spp.* in animal feed samples by PCR after culture enrichment." *Appl Environ Microbiol* **70**(1): 69-75.
- Lövenklev, M. (2003). A molecular approach for investigation of the prevalence and neurotoxin formation of *Clostridium botulinum* in food safety assessment. Applied Microbiology. Lund, Lund Institute of Technology.
- Lövenklev, M., E. Holst, E. Borch and P. Rådström (2004). "Relative Neurotoxin Gene Expression in *Clostridium botulinum* Type B, Determined Using Quantitative Reverse Transcription-PCR." *Appl. Environ. Microbiol.* **70**(5): 2919-2927.

- Malorny, B., P. T. Tassios, P. Rådström, N. Cook, M. Wagner and J. Hoorfar (2003). "Standardization of diagnostic PCR for the detection of foodborne pathogens." *Int J Food Microbiol* **83**(1): 39-48.
- Marshall, K. M., M. Bradshaw, S. Pellett and E. A. Johnson (2007). "Plasmid encoded neurotoxin genes in *Clostridium botulinum* serotype A subtypes." *Biochem Biophys Res Commun* **361**(1): 49-54.
- Marvaud, J. C., M. Gibert, K. Inoue, Y. Fujinaga, K. Oguma and M. R. Popoff (1998). "botR/A is a positive regulator of botulinum neurotoxin and associated non-toxin protein genes in *Clostridium botulinum* A." *Mol Microbiol* **29**(4): 1009-18.
- Mathews, S. A., K. M. Volp and P. Timms (1999). "Development of a quantitative gene expression assay for *Chlamydia trachomatis* identified temporal expression of sigma factors." *FEBS Lett* **458**(3): 354-8.
- McLauchlin, J., K. A. Grant and C. L. Little (2006). "Food-borne botulism in the United Kingdom." *J Public Health (Oxf)* **28**(4): 337-42.
- Meng, X., T. Karasawa, K. Zou, X. Kuang, X. Wang, C. Lu, C. Wang, K. Yamakawa and S. Nakamura (1997). "Characterization of a neurotoxigenic *Clostridium butyricum* strain isolated from the food implicated in an outbreak of food-borne type E botulism." *J Clin Microbiol* **35**(8): 2160-2.
- Meusburger, S., S. Reichert, S. Heibl, M. Nagl, F. Karner, I. Schachinger and F. Allerberger (2006). "Outbreak of foodborne botulism linked to barbecue, Austria, 2006." *Euro Surveill* **11**(12): E061214 4.
- Midura, T. F. (1996). "Update: infant botulism." *Clin Microbiol Rev* **9**(2): 119-25.
- Mitchell, P. A. and P. T. Pons (2001). "Wound botulism associated with black tar heroin and lower extremity cellulitis." *J Emerg Med* **20**(4): 371-5.

- Moncrief, J. S., L. A. Barroso and T. D. Wilkins (1997). "Positive regulation of *Clostridium difficile* toxins." *Infect Immun* **65**(3): 1105-8.
- Monpoeho, S., M. Coste-Burel, M. Costa-Mattioli, B. Besse, J. J. Chomel, S. Billaudel and V. Ferre (2002). "Application of a real-time polymerase chain reaction with internal positive control for detection and quantification of enterovirus in cerebrospinal fluid." *Eur J Clin Microbiol Infect Dis* **21**(7): 532-6.
- Morse, D. L., L. K. Pickard, J. J. Guzewich, B. D. Devine and M. Shayegani (1990). "Garlic-in-oil associated botulism: episode leads to product modification." *Am J Public Health* **80**(11): 1372-3.
- Mullis, K. B. and F. A. Faloona (1987). "Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction." *Methods Enzymol* **155**: 335-50.
- Nagasawa, T., K. Tanizawa, T. Satoda and H. Yamada (1988). "Diaminopropionate ammonia-lyase from *Salmonella typhimurium*. Purification and characterization of the crystalline enzyme, and sequence determination of the pyridoxal 5'-phosphate binding peptide." *J Biol Chem* **263**(2): 958-64.
- Neumaier, M., A. Braun and C. Wagener (1998). "Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics. International Federation of Clinical Chemistry Scientific Division Committee on Molecular Biology Techniques." *Clin Chem* **44**(1): 12-26.
- Nevas, M., M. Lindstrom, A. Virtanen, S. Hielm, M. Kuusi, S. S. Arnon, E. Vuori and H. Korkeala (2005). "Infant botulism acquired from household dust presenting as sudden infant death syndrome." *J Clin Microbiol* **43**(1): 511-3.
- Niesters, H. G. (2004). "Molecular and diagnostic clinical virology in real time." *Clin Microbiol Infect* **10**(1): 5-11.
- Noonan, K. E. and I. B. Roninson (1988). "mRNA phenotyping by enzymatic amplification of randomly primed cDNA." *Nucleic Acids Res* **16**(21): 10366.

- Obiero, J., S. A. Bonderoff, M. M. Goertzen and D. A. Sanders (2006). "Expression, purification, crystallization and preliminary X-ray crystallographic studies of *Deinococcus radiodurans* thioredoxin reductase." *Acta Crystallograph Sect F Struct Biol Cryst Commun* **62**(Pt 8): 757-60.
- Ogert, R. A., J. Edward Brown, B. R. Singh, L. C. Shriver-Lake and F. S. Ligler (1992). "Detection of *Clostridium botulinum* toxin A using a fiber optic-based biosensor." *Analytical Biochemistry* **205**(2): 306-312.
- Oliveira, M. A., K. F. Discola, S. V. Alves, J. A. Barbosa, F. J. Medrano, L. E. Netto and B. G. Guimaraes (2005). "Crystallization and preliminary X-ray diffraction analysis of NADPH-dependent thioredoxin reductase I from *Saccharomyces cerevisiae*." *Acta Crystallograph Sect F Struct Biol Cryst Commun* **61**(Pt 4): 387-90.
- Passaro, D. J., S. B. Werner, J. McGee, W. R. Mac Kenzie and D. J. Vugia (1998). "Wound botulism associated with black tar heroin among injecting drug users." *Jama* **279**(11): 859-63.
- Perelle, S., F. Dilasser, B. Malorny, J. Grout, J. Hoorfar and P. Fach (2004). "Comparison of PCR-ELISA and LightCycler real-time PCR assays for detecting *Salmonella spp.* in milk and meat samples." *Mol Cell Probes* **18**(6): 409-20.
- Peruski, A. H., L. H. Johnson, 3rd and L. F. Peruski, Jr. (2002). "Rapid and sensitive detection of biological warfare agents using time-resolved fluorescence assays." *J Immunol Methods* **263**(1-2): 35-41.
- Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." *Nucleic Acids Res* **29**(9): E45-E45.
- Poulet, S., D. Hauser, M. Quanz, H. Niemann and M. R. Popoff (1992). "Sequences of the botulinal neurotoxin E derived from *Clostridium botulinum* type E (strain Beluga) and *Clostridium butyricum* (strains ATCC 43181 and ATCC 43755)." *Biochem Biophys Res Commun* **183**(1): 107-13.

- Poulstrup, A., A. T. Nielsen, M. Binder, K. Moller, M. Moller, J. Nielsen and H. L. Hansen (1999). "[Botulism caused by a commercially produced product]." *Ugeskr Laeger* **161**(19): 2815-6.
- Pourshafie, M. R., M. Saifie, A. Shafiee, P. Vahdani, M. Aslani and J. Salemian (1998). "An outbreak of food-borne botulism associated with contaminated locally made cheese in Iran." *Scand J Infect Dis* **30**(1): 92-4.
- Prévot, A. R. (1953). "Rapport d'introduction du président du sous-comite *Clostridium* pour l'unification de la nomenclature des types toxigéniques de *C. botulinum*." *Int. Bull. Bact. Nomencl.* **3**: 120-123.
- Proulx, J. F., V. Milor-Roy and J. Austin (1997). "Four outbreaks of botulism in Ungava Bay, Nunavik, Quebec." *Can Commun Dis Rep* **23**(4): 30-2.
- Raffestin, S., J. C. Marvaud, R. Cerrato, B. Dupuy and M. R. Popoff (2004). "Organization and regulation of the neurotoxin genes in *Clostridium botulinum* and *Clostridium tetani*." *Anaerobe Molecular Biology and Pathogenesis of the Clostridia. Special topics from the Fourth International Conference on the Molecular Biology and Pathogenesis of the Clostridia (CllostPath 2003)* **10**(2): 93-100.
- Rajeevan, M. S., D. G. Ranamukhaarachchi, S. D. Vernon and E. R. Unger (2001). "Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies." *Methods* **25**(4): 443-51.
- Rappolee, D. A., D. Mark, M. J. Banda and Z. Werb (1988). "Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping." *Science* **241**(4866): 708-12.
- Rappolee, D. A., A. Wang, D. Mark and Z. Werb (1989). "Novel method for studying mRNA phenotypes in single or small numbers of cells." *J Cell Biochem* **39**(1): 1-11.

- Reynisson, E., M. H. Josefsen, M. Krause and J. Hoorfar (2006). "Evaluation of probe chemistries and platforms to improve the detection limit of real-time PCR." *J Microbiol Methods* **66**(2): 206-16.
- Rivera, V. R., F. J. Gamez, W. K. Keener, J. A. White and M. A. Poli (2006). "Rapid detection of *Clostridium botulinum* toxins A, B, E, and F in clinical samples, selected food matrices, and buffer using paramagnetic bead-based electrochemiluminescence detection." *Anal Biochem* **353**(2): 248-56.
- Rodriguez-Lazaro, D., M. D'Agostino, M. Pla and N. Cook (2004). "Construction strategy for an internal amplification control for real-time diagnostic assays using nucleic Acid sequence-based amplification: development and clinical application." *J Clin Microbiol* **42**(12): 5832-6.
- Rosetti, F., E. Castelli, J. Labbé and R. Funes (1999). "Outbreak of Type E Botulism Associated with Home-cured Ham Consumption." *Anaerobe* **5**: 171-172.
- Ross, R. A. and A. B. Onderdonk (2000). "Production of toxic shock syndrome toxin 1 by *Staphylococcus aureus* requires both oxygen and carbon dioxide." *Infect Immun* **68**(9): 5205-9.
- Rådström, P., R. Knutsson, P. Wolffs, M. Lövenklev and C. Löfström (2004). "Pre-PCR processing: strategies to generate PCR-compatible samples." *Mol Biotechnol* **26**(2): 133-46.
- Sachse, K. (2003). "Specificity and performance of diagnostic PCR assays." *Methods Mol Biol* **216**: 3-29.
- Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B. R. DasGupta and C. Montecucco (1992). "Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin." *Nature* **359**(6398): 832-5.

- Schiavo, G., C. Malizio, W. S. Trimble, P. Polverino de Laureto, G. Milan, H. Sugiyama, E. A. Johnson and C. Montecucco (1994). "Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond." *J Biol Chem* **269**(32): 20213-6.
- Schiavo, G., O. Rossetto, A. Santucci, B. R. DasGupta and C. Montecucco (1992). "Botulinum neurotoxins are zinc proteins." *J Biol Chem* **267**(33): 23479-83.
- Schiavo, G., A. Santucci, B. R. Dasgupta, P. P. Mehta, J. Jontes, F. Benfenati, M. C. Wilson and C. Montecucco (1993a). "Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds." *FEBS Lett* **335**(1): 99-103.
- Schiavo, G., C. C. Shone, M. K. Bennett, R. H. Scheller and C. Montecucco (1995). "Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins." *J Biol Chem* **270**(18): 10566-70.
- Schiavo, G., C. C. Shone, O. Rossetto, F. C. Alexander and C. Montecucco (1993b). "Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin." *J Biol Chem* **268**(16): 11516-9.
- Schmittgen, T. D. and B. A. Zakrajsek (2000). "Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR." *J Biochem Biophys Methods* **46**(1-2): 69-81.
- Schwarz, G. and R. R. Mendel (2006). "Molybdenum cofactor biosynthesis and molybdenum enzymes." *Annu Rev Plant Biol* **57**: 623-47.
- Sebahia, M., M. W. Peck, N. P. Minton, N. R. Thomson, M. T. Holden, W. J. Mitchell, A. T. Carter, S. D. Bentley, D. R. Mason, L. Crossman, C. J. Paul, A. Ivens, M. H. Wells-Bennik, I. J. Davis, A. M. Cerdeno-Tarraga, C. Churcher, M. A. Quail, T. Chillingworth, T. Feltwell, A. Fraser, I. Goodhead, Z. Hance, K. Jagels, N. Larke, M. Maddison, S. Moule, K. Mungall, H. Norbertczak, E. Rabinowitsch, M. Sanders, M. Simmonds, B. White, S. Whithead and J. Parkhill (2007). "Genome sequence of a

- proteolytic (Group I) *Clostridium botulinum* strain Hall A and comparative analysis of the clostridial genomes." *Genome Res* **17**(7): 1082-92.
- Shan, X. C., P. Wolffs and M. W. Griffiths (2005). "Rapid and quantitative detection of hepatitis A virus from green onion and strawberry rinses by use of real-time reverse transcription-PCR." *Appl Environ Microbiol* **71**(9): 5624-6.
- Shapiro, B. E., O. Soto, S. Shafqat and H. Blumenfeld (1997). "Adult botulism." *Muscle Nerve* **20**(1): 100-2.
- Shapiro, R. L., C. Hatheway and D. L. Swerdlow (1998). "Botulism in the United States: a clinical and epidemiologic review." *Ann Intern Med* **129**(3): 221-8.
- Sharkey, F. H., S. I. Markos and R. W. Haylock (2004). "Quantification of toxin-encoding mRNA from *Clostridium botulinum* type E in media containing sorbic acid or sodium nitrite by competitive RT-PCR." *FEMS Microbiol Lett* **232**(2): 139-44.
- Sharma, S. K., B. S. Eblen, R. L. Bull, D. H. Burr and R. C. Whiting (2005). "Evaluation of lateral-flow *Clostridium botulinum* neurotoxin detection kits for food analysis." *Appl Environ Microbiol* **71**(7): 3935-41.
- Sheridan, R. E., T. J. Smith and M. Adler (2005). "Primary cell culture for evaluation of botulinum neurotoxin antagonists." *Toxicon* **45**(3): 377-82.
- Shimamura, T., S. Watanabe and S. Sasaki (1985). "Enhancement of enterotoxin production by carbon dioxide in *Vibrio cholerae*." *Infect Immun* **49**(2): 455-6.
- Shin, N. R., J. H. Shin, J. H. Chun, S. Y. Yoon, B. S. Kim, H. B. Oh and G. E. Rhie (2006). "Determination of neurotoxin gene expression in *Clostridium botulinum* type A by quantitative RT-PCR." *Mol Cells* **22**(3): 336-42.
- Sieradzan, K. A. (2005). "Wound Botulism." *Practical Neurology* **5**(1): 46-52.

- Smith, T. J., J. Lou, I. N. Geren, C. M. Forsyth, R. Tsai, S. L. Laporte, W. H. Tepp, M. Bradshaw, E. A. Johnson, L. A. Smith and J. D. Marks (2005). "Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization." *Infect Immun* **73**(9): 5450-7.
- Sobel, J. (2005). "Botulism." *Clin Infect Dis* **41**(8): 1167-73.
- Sobel, J., M. Malavet and S. John (2007). "Outbreak of clinically mild botulism type E illness from home-salted fish in patients presenting with predominantly gastrointestinal symptoms." *Clin Infect Dis* **45**(2): e14-6.
- Solomon, H. M., E. J. Rhodehamel and D. A. Kautter (1998). "Growth and toxin production by *Clostridium botulinum* on sliced raw potatoes in a modified atmosphere with and without sulfite." *J Food Prot* **61**(1): 126-8.
- Song, L., S. Ahn and D. R. Walt (2006). "Fiber-optic microsphere-based arrays for multiplexed biological warfare agent detection." *Anal Chem* **78**(4): 1023-33.
- Sonnabend, O. A., W. F. Sonnabend, U. Krech, G. Molz and T. Sigrist (1985). "Continuous microbiological and pathological study of 70 sudden and unexpected infant deaths: toxigenic intestinal *Clostridium botulinum* infection in 9 cases of sudden infant death syndrome." *Lancet* **1**(8423): 237-41.
- Spiegelman, S., K. F. Watson and D. L. Kacian (1971). "Synthesis of DNA complements of natural RNAs: a general approach." *Proc Natl Acad Sci U S A* **68**(11): 2843-5.
- Stretton, S. and A. E. Goodman (1998). "Carbon dioxide as a regulator of gene expression in microorganisms." *Antonie Van Leeuwenhoek* **73**(1): 79-85.
- Stöcher, M., V. Leb, G. Hölzl and J. Berg (2002). "A simple approach to the generation of heterologous competitive internal controls for real-time PCR assays on the LightCycler." *J Clin Virol* **25 Suppl 3**: S47-53.

- Suen, J. C., C. L. Hatheway, A. G. Steigerwalt and D. J. Brenner (1988b). "Genetic confirmation of identities of neurotoxicogenic *Clostridium baratii* and *Clostridium butyricum* implicated as agents of infant botulism." J Clin Microbiol **26**(10): 2191-2.
- Suen, J. G., C. L. Hatheway, A. G. Steigerwalt and D. J. Brenner (1988a). "*Clostridium argentinense* sp. nov.: a genetically homogenous group composed of all strains of *Clostridium botulinum* toxin type G and some non-toxicogenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*." Int. J. Syst. Bacteriol. **38**: 375-381.
- Sutton, R. B., D. Fasshauer, R. Jahn and A. T. Brunger (1998). "Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution." Nature **395**(6700): 347-53.
- Szabo, E. A., J. M. Pemberton and P. M. Desmarchelier (1993). "Detection of the genes encoding botulinum neurotoxin types A to E by the polymerase chain reaction." Appl Environ Microbiol **59**(9): 3011-20.
- Takeshi, K., Y. Fujinaga, K. Inoue, H. Nakajima, K. Oguma, T. Ueno, H. Sunagawa and T. Ohyama (1996). "Simple method for detection of *Clostridium botulinum* type A to F neurotoxin genes by polymerase chain reaction." Microbiol Immunol **40**(1): 5-11.
- Temin, H. M. (1964). "Homology between Rna from Rous Sarcoma Virus and DNA from Rous Sarcoma Virus-Infected Cells." Proc Natl Acad Sci U S A **52**: 323-9.
- Temin, H. M. and S. Mizutani (1970). "RNA-dependent DNA polymerase in virions of Rous sarcoma virus." Nature **226**(5252): 1211-3.
- Thompson, D. E., J. K. Brehm, J. D. Oultram, T. J. Swinfield, C. C. Shone, T. Atkinson, J. Melling and N. P. Minton (1990). "The complete amino acid sequence of the *Clostridium botulinum* type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene." Eur J Biochem **189**(1): 73-81.

- Toouli, C. D., D. R. Turner, S. A. Grist and A. A. Morley (2000). "The effect of cycle number and target size on polymerase chain reaction amplification of polymorphic repetitive sequences." *Anal Biochem* **280**(2): 324-6.
- Townes, J. M., P. R. Cieslak, C. L. Hatheway, H. M. Solomon, J. T. Holloway, M. P. Baker, C. F. Keller, L. M. McCroskey and P. M. Griffin (1996). "An outbreak of type A botulism associated with a commercial cheese sauce." *Ann Intern Med* **125**(7): 558-63.
- Turton, K., J. A. Chaddock and K. R. Acharya (2002). "Botulinum and tetanus neurotoxins: structure, function and therapeutic utility." *Trends Biochem Sci* **27**(11): 552-8.
- Tyagi, S. and F. R. Kramer (1996). "Molecular beacons: probes that fluoresce upon hybridization." *Nat Biotechnol* **14**(3): 303-8.
- Uo, T., T. Yoshimura, T. Nishiyama and N. Esaki (2002). "Gene cloning, purification, and characterization of 2,3-diaminopropionate ammonia-lyase from *Escherichia coli*." *Biosci Biotechnol Biochem* **66**(12): 2639-44.
- Walker, N. J. (2001). "Real-time and quantitative PCR: applications to mechanism-based toxicology." *J Biochem Mol Toxicol* **15**(3): 121-7.
- van Ermengem, E. (1979). "Classics in infectious diseases. A new anaerobic bacillus and its relation to botulism. E. van Ermengem. Originally published as "Ueber einen neuen anaeroben Bacillus und seine Beziehungen zum Botulismus" in *Zeitschrift für Hygiene und Infektionskrankheiten* 26: 1-56, 1897." *Rev Infect Dis* **1**(4): 701-19.
- Whelan, S. M., M. J. Elmore, N. J. Bodsworth, J. K. Brehm, T. Atkinson and N. P. Minton (1992). "Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of its entire nucleotide sequence." *Appl Environ Microbiol* **58**(8): 2345-54.
- Whiting, R. C. and K. A. Naftulin (1992). "Effect of headspace oxygen concentration on growth and toxin production by proteolytic strains of *Clostridium botulinum*." *Journal of Food Protection* **55**(1): 23-27.

- Wictome, M., K. Newton, K. Jameson, B. Hallis, P. Dunnigan, E. Mackay, S. Clarke, R. Taylor, J. Gaze, K. Foster and C. Shone (1999). "Development of an in vitro bioassay for *Clostridium botulinum* type B neurotoxin in foods that is more sensitive than the mouse bioassay." *Appl Environ Microbiol* **65**(9): 3787-92.
- Wittwer, C. T., M. G. Herrmann, A. A. Moss and R. P. Rasmussen (1997). "Continuous fluorescence monitoring of rapid cycle DNA amplification." *Biotechniques* **22**(1): 130-1, 134-8.
- Wolffs, P. (2004). Pre-PCR processing strategies for quantitative detection of food-borne pathogens using real-time PCR. *Applied Microbiology*. Lund, Lund Institute of Technology.
- Wolffs, P., H. Grage, O. Hagberg and P. Rådström (2004a). "Impact of DNA polymerases and their buffer systems on quantitative real-time PCR." *J Clin Microbiol* **42**(1): 408-11.
- Wolffs, P., R. Knutsson, B. Norling and P. Rådström (2004b). "Rapid quantification of *Yersinia enterocolitica* in pork samples by a novel sample preparation method, flotation, prior to real-time PCR." *J Clin Microbiol* **42**(3): 1042-7.
- Wolffs, P., B. Norling, J. Hoorfar, M. Griffiths and P. Rådström (2005). "Quantification of *Campylobacter spp.* in chicken rinse samples by using flotation prior to real-time PCR." *Appl Environ Microbiol* **71**(10): 5759-64.
- Wu, H. C., Y. L. Huang, S. C. Lai, Y. Y. Huang and M. F. Shaio (2001). "Detection of *Clostridium botulinum* neurotoxin type A using immuno-PCR." *Lett Appl Microbiol* **32**(5): 321-5.
- Yamasaki, S., A. Baumeister, T. Binz, J. Blasi, E. Link, F. Cornille, B. Roques, E. M. Fykse, T. C. Sudhof, R. Jahn and et al. (1994). "Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinical neurotoxins and tetanus toxin." *J Biol Chem* **269**(17): 12764-72.

- Yoon, S. Y., G. T. Chung, D. H. Kang, C. Ryu, C. K. Yoo and W. K. Seong (2005). "Application of real-time PCR for quantitative detection of *Clostridium botulinum* type A toxin gene in food." *Microbiol Immunol* **49**(6): 505-11.
- Zhou, Y., H. Sugiyama, H. Nakano and E. A. Johnson (1995). "The genes for the *Clostridium botulinum* type G toxin complex are on a plasmid." *Infect Immun* **63**(5): 2087-91.
- Öberg, S. (1994). "Botulism." *Epid-aktuellt* **17**(8): 5.